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GENETIC ETIOLOGICAL STUDY OF INTELLECTUAL DISABILITY IN DR CONGO

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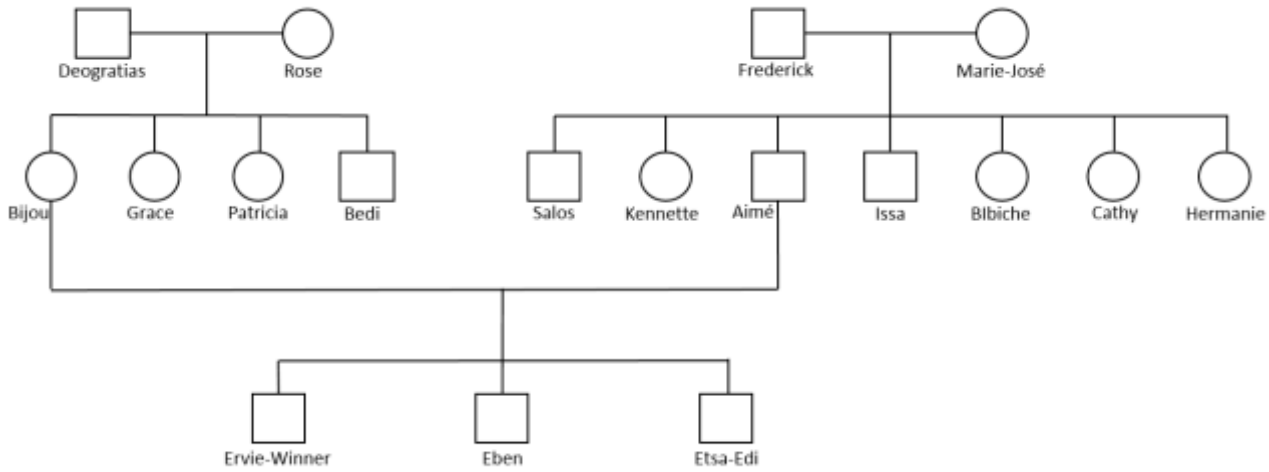
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LIST OF ABBREVIATIONS

5'-UTR	5-prime Untranslated Region
ADHD	Attention Deficit and Hyperactivity Disorder
AGG	Adenine-Guanine-Guanine
AHC	Adrenal Hypoplasia Congenita
ALZ	Aimé Lumaka Zola
Array-CGH	Microarray-Comparative Genome Hybridization
ASD	Autism Spectrum Disorders
Bp	Base Pair
CAH	Congenital Adrenal Hyperplasia
CDC	Centres for Disease Control
CGG	Cytosine-Guanine-Guanine
CHD	Congenital Heart Disease
CNPP	Centre de Neuro-PsychoPathologie de Kinshasa
CNV	Copy Number Variation
CUK	Cliniques Universitaires de Kinshasa
DECIPHER	Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources
DGV	Database of Genomic Variants
DRC and DR Congo	Democratic Republic of Congo
DS	Down syndrome
Eben	Eternel nous a BENi
Ervie	Eternel Règne dans nos VIE
Etsa	Eternel nous Surpris Agréablement
FISH	Fluorescent in situ Hybridization
FMR1	Fragile X Mental Retardation 1
FX-	Fragile X negative
FX+	Fragile X positive
FXS	Fragile X Syndrome
HH	Hypogonadotropic Hypogonadism
ID	Intellectual Disability
Indels	Insertions/deletions
INRB	Institut National de recherche Biomédicale
IQ	Intelligence Quotient
Kb	Kilo-bases
KD	Koen Devriendt
KU Leuven	Katholieke Universiteit Leuven
M.H.	Maureen Holvoet
Mb	Megabase
MIM or OMIM	Online Mendelian Inheritance in Man
NGS	Next-Generation Sequencing
OAVS	Oculo-Auriculo-Vertebral Spectrum
OFC	Occipital-Frontal Circumference

PCR	Polymerase Chain Reaction
PLT	Prosper Lukusa Tshilobo
qPCR	quantitative Polymerase Chain Reaction
SD	Standard Deviation
SMS	Smith-Magenis Syndrome
SNV	Single Nucleotide Variant
UNIKIN	Université de Kinshasa
WBS	Williams-Beuren Syndrome
WES	Whole Exome Sequencing
XCI	X-Chromosome Inactivation assay
XLID	X-linked Intellectual Disability

INTRODUCTION

1.1 Definitions

Intellectual disability (ID) is defined as a significant impairment of cognitive functions or intelligence and adaptive functions with onset before the age of 18 years (Maes, Fryns et al. 2000; Rauch, Hoyer et al. 2006; Tasse 2009). Many terms have been proposed to designate this impairment of mental health. Of these, intellectual disability (ID) is currently widely accepted to replace the previous terms mental retardation (MR), learning disabilities or intellectual impairment (Salvador-Carulla and Bertelli 2008).

Intelligence can be understood as the ability to reason, solve problems and to learn. Intelligence integrates functions such as perception, attention, memory, language or planning. As a potential, intelligence is reliably measured by standardized tests and expressed in a numeral score named Intelligence Quotient (IQ) (Berkenstadt, Ries-Levavi et al. 2007). Statistically, normal intelligence is defined by the mean ± 2SD, or an IQ of 100 ± 30. However, individuals with an IQ between 70 and 85 are considered to have border-line intelligence, since they also experience impairments. IQ scores between 50 and 70 are defined as mild ID, between 50 and 35 as moderate ID, between 35 and 20 as severe ID and finally, below 20 as profound ID (http://www.who.int/whr/2001/en/whr01_en.pdf). It is estimated that about 3 % of world population have an IQ below 70 and 1/200 below 50 (Vissers, de Vries et al. 2010).

Adaptive skills refer to basic skills needed for everyday life. They include communication, self-care, home living, social skills, leisure, health and safety, self-direction, functional academics (reading, writing, basic mathematics), and work (Hagerman, Amiri et al. 1991).

Many instruments or standardized tests have been designed to assess IQ. These are known as psychometric tests. The majority of them include images or pictograms to evaluate the ability to cope with daily live. Surprisingly, most of the existing tools has been validated in Western countries and the pictograms typically refer to daily situations in these countries. Children from countries such as the DR Congo may perform poorly on such items when assessed using such culturally inappropriate tools and this may lead to an underestimation of their intelligence. Therefore, efforts are needed to produce assessment tools adapted to Congolese children.

1.2 Impact of ID

ID has a major impact on the individual, his family and the communities. Once the ID is established, it will cause psychological and financial burden that will have a further negative effect on social development (Ropers 2010). At the individual and familial level, ID may psychologically impact relatives. Abasiubong et al., investigated psychological effects of ID on mothers in Nigeria and reported high levels of anxiety in 25.5 % and depression in 10.4 % (Abasiubong, Obembe et al. 2006). In another study, they reported that 2.8 % thought about the physical elimination of their affected child (Abasiubong, Obembe et al. 2008). In addition to psychological damage, ID of a child may increase family expenses up to 8 % (Rauch, Hoyer et al. 2006).

There is a vicious circle, particularly in developing countries, involving poverty and ID. Special cares to ID patients can be prohibitive as proven in developing countries (Wang 2012; Buescher, Cidav et al. 2014; Genereaux, van Karnebeek et al. 2015). Therefore, the family can be quickly run out of finances in limited resources countries, were national responses are limited. This results in increased poverty which has as corollaries malnutrition, limited access to healthcare, infectious diseases. It is well known that these corollaries are among environmental factors causing ID.

1.3 The aetiology of Intellectual disability

ID refers to a characteristic observed in a heterogeneous group of disorders. Consequently, causes of ID are also heterogeneous. The search for the cause of ID is often a complex process, and involves detailed family and personal history, clinical examination with special attention for the presence of dysmorphic signs and complementary testing, such as brain imaging, metabolic and genetic tests (Moeschler and Shevell 2014).

Various way of classifying the aetiology of ID have been proposed, depending on the point of view and purpose of the classification (Chelly, Khelfaoui et al. 2006; Rauch, Hoyer et al. 2006). Causes of ID may be classified according to the time of origin (pre-, peri-, and postnatal), or according to the type of defect (genetic, environmental, and multifactorial). A classification including both the timing and type of defect may also be considered (Moog 2005). In Western countries, genetic causes account for an estimated 4 to 41.1 % of cases (Battaglia, Bianchini et al. 1999; Moeschler 2008; Bernardini, Alesi et al. 2010).

1.4 Approaches to identify genetic causes: targeted or genome-wide screening

From a clinical point of view, ID can be isolated or associated with additional dysmorphic, neurological and behavioural features, defining specific syndromes such as Down syndrome, Partington syndrome or Smith-Magenis syndrome. These syndromes present a recognizable pattern of malformation with the presence of multiple minor anomalies (called dysmorphism) and/or major malformations. This is often associated with a behavioural phenotype (Harris 2010). When a syndrome is identified, targeted testing is possible meaning the analysis of a specific gene or performance of a metabolic test. Hence, the clinical recognition of specific disorders, especially when rare, is a critical step in the diagnostic process.

1.4.1 Syndromology

Syndromology is the medical discipline with expertise in the classification and diagnosis of specific syndromes. During the last decades, tremendous efforts have been made in clinically describing and defining a large number of syndromes in large cohorts of patients. Syndrome databases have been implemented such as the London Medical Dysmorphology Database and POSSUM to offer the possibility to match combinations of features to syndromes (van Steensel and Winter 1998). Tools for an accurate description of phenotypic features have been developed including an internationally accepted Human Phenotype Ontology (Robinson, Kohler et al. 2008) and a series of articles with the definition and illustration of a wide range of morphological anomalies (Allanson, Cunniff et al. 2009). Efforts to introduce objective morphometric analysis of digital images (2D or 3D) of patients are underway, and hold great promise. However, syndromology remains largely based on expert-opinion, especially for rare syndromes.

1.4.2 Genetic testing

Over the last 10 years, progress in the identification of genetic causes of ID in Western countries has been impressive. This is essentially driven by the technological revolutions such as microarray-Comparative Genome Hybridisation (array-CGH) and more recently Next-Generation Sequencing (NGS) technology. These techniques allow a genome-wide screening for mutations of different sizes, copy number variations (larger than 1 kb), single nucleotide variants (SNV's, 1 nucleotide) and indels (in between these two). This has accelerated the identification of genes causing previously well characterized genetic disorders as well as the recognition of many novel entities. This is illustrated by the identification of submicroscopic chromosomal imbalances causing common and well known syndromes such as Williams syndrome (del7q11.23), Velocardiofacial syndrome (del22q11.2), Smith-Magenis syndrome (del17p11.2). Novel and now also clinically recognizable syndromes include del17q21.31, del1p36 and the 7q11.23 duplication (Nevado, Mergener et al. 2014).

For monogenic disorders, the progress has been even more impressive in terms of the number of genes identified. The causal gene can now often be identified through the analysis of only a few families with an (ultra)rare disorder, resulting in a spectacular increase in the number of novel entities and associated genes (Bamshad, Ng et al. 2011; Wright, Fitzgerald et al. 2015). Moreover, knowledge gained is rapidly translated into the clinic, thus revolutionizing genetic diagnosis.

In particular, array-CGH allows a genome-wide screening for chromosomal imbalances at a resolution exceeding at least 10 fold that of traditional karyotyping. On average, the detection rate of a pathogenic copy number variation (CNV) in an individual with clinically unexplained ID is 15-20 %, compared to approximately 3 % by traditional karyotyping (Miller, Adam et al. 2010). The likelihood of detecting a causal chromosomal imbalance depends on the phenotype. The presence of a major malformation (e.g. of the heart, musculoskeletal system or central nervous system) is associated with a higher chance of detecting a pathogenic CNV (Shoukier, Klein et al. 2013; Preiksaitiene, Molyte et al. 2014). In a study of children with syndromic congenital heart defects (CHD), the presence of dysmorphism (defined as three or more minor anomalies) increased the likelihood of detecting a pathogenic chromosomal imbalance (Breckpot, Thienpont et al. 2010). Battaglia also reported a higher diagnostic yield in ID when associated with dysmorphism (Battaglia, Doccini et al. 2013). Array-CGH has been applied for other indications besides ID, but in general, the yield of pathogenic CNV's is lower. For instance, in individuals with an isolated CHD, the yield varied between 4 to 14 % (Andersen, Troelsen Kde et al. 2014). In autism spectrum disorders (ASD), two studies using a platform with a resolution of 20 kb or larger, detected de novo CNV's in 8 % of cases, 6 % higher than in their unaffected siblings (Levy, Ronemus et al. 2011; Sanders, Ercan-Sencicek et al. 2011). Also in ASD, more de novo CNV's were detected in individuals with ID compared to those with a normal IQ (Sanders, Ercan-Sencicek et al. 2011). Array-CGH studies in epilepsy have a yield of clinically relevant CNV's of approximately 12 % (Sisodiya 2015). Also in schizophrenia CNV's appear to play a pathogenic role in a variable subset of patients (Basset et al., 2010).

Over the past 10 years, many novel pathogenic CNV's have been identified causing various developmental disorders. The clinical utility of Array-CGH is complicated by the fact that several of these CNV's are in fact susceptibility loci, associated with an increased risk of a disorder. This is reflected by the fact that even though they may occur de novo, they are often inherited from an unaffected parent. Moreover, these CNV's are associated with variable clinical manifestations. For instance, the deletion in chromosome 16p11.2 is a risk factor for ID, autism spectrum disorders and epilepsy, and is associated with obesity and macrocephaly (Miller, Nasir et al. 1993). In the genetic clinic, where risk prediction is one of the main tasks, the finding of such a CNV's poses major challenges. This is especially true in a prenatal setting (Vanakker, Vilain et al. 2014).

Until recently, testing for monogenic conditions was limited to specific genes, guided by a clinical (suspected) diagnosis. Recently, NGS technology allows the massive parallel sequencing of a large number of candidate genes for a certain phenotype (gene panels), the exons of all genes associated to a Mendelian disorder (Mendeliome), the exons of all known genes (the exome) and even the entire genome (whole genome sequencing). Despite technical limitations, which explain the lower sensitivity to detect a mutation compared to traditional Sanger sequencing, this approach offers unprecedented opportunities for mutation screening in individuals with a heterogeneous condition such as ID. In large clinically preselected cohorts, the additional yield of exome analysis in a trio approach (both parents and child) ranged from 16 % to 45 % (Rauch, Wieczorek et al. 2012; Yang, Muzny et al. 2013; Wright, Fitzgerald et al. 2015). More recently, using whole genome sequencing, an etiological diagnosis could be reached in an additional 42 % of individuals (Gilissen, Hehir-Kwa et al. 2014). Taken together, in clinically undiagnosed patients, it is estimated that the cumulative chance of reaching an etiological diagnosis using the most advanced genetic testing (i.e. Array-CGH and whole genome sequencing in a trio approach) is approximately 62 % (Gilissen, Hehir-Kwa et al. 2014). The aetiology of the ID in the remaining unsolved cases is currently not known. Acquired, non-genetic causes may involve unidentified environmental insults disturbing brain development. One can also envisage the occurrence of different types of other genetic mutations that escape the detection of current tools, including mosaicism, non-coding mutations, certain structural variations and the possibility of epigenetic mutations. Most strategies are currently biased towards the identification of de novo mutations or those absent in a normal control population. The consequences of inherited mutations with variable expression due to imprinting or due to additional oligogenic modifiers (comparable to the susceptibility CNV's) are only beginning to be investigated.

Despite remarkable progress, many cases remain without a precise etiological diagnosis. Reasons include the enormous heterogeneity in causes of ID or in clinically defined conditions (such as epileptic encephalopathy), the lack of clinically recognizable phenotypes for many genetic mutations and the variability in expression. This frequently results in a long diagnostic process, involving many consecutive rounds of (genetic) testing, often without reaching a definite etiological diagnosis. This "diagnostic odyssey" causes much distress in the families and may result in a diagnostic fatigue, both for the parents and physicians (Carmichael, Tsipis et al. 2015). One consequence of this is that many older individuals with ID often have not been investigated recently with up-to-date knowledge and technology. This group often comes back to the attention of the clinical geneticist when siblings raise questions regarding recurrence risk for their own children. As a result, for many rare and more recently delineated conditions, the adult phenotype is not known, hampering adequate counselling in young children.

1.4.3 Data interpretation: normal, pathogenic or unclassified?

A diagnostic test based on genome wide screening for CNV's, indels or SNV's is challenging regarding the interpretation of the data. Genomic studies have indeed revealed an unanticipated amount of variation in the normal population. Thus, one can only speak of a reference genome: the "normal genome" does not exist. As a result, extensive filtering of CNV and sequence data is required in order to interpret the data. Below, we describe the rationale behind the different filter steps.

Since many variants are common in the normal population, a first step is the removal of known, common variants. There exist several databases collecting normal population variants (Richards, Aziz et al. 2015). Since many variants occur at an increased frequency in specific population, additional filtering against a local database of local common variants is an effective step (Brown, Lee et al. 2015). Under the hypothesis of a de novo cause of the ID (which is the most common situation), the causal variants are not expected to be observed in the normal population. Taken possible errors in the database into account (e.g. sequencing errors), one excludes variants that are observed in a significant percentage of the normal population. For X-linked and autosomal recessive conditions, one needs to consider that these variants may be present in normal carriers. In (rare) founder populations, where pathogenic variants may reach higher frequencies, one needs to adapt these limits.

The databases of known polymorphic variants are complemented by disease specific mutation databases (Richards, Aziz et al. 2015). However, most databases are contaminated with a large amount of variants that are in fact non-functional variants. For instance, for cardiomyopathies and arrhythmias, in exome sequences from normal persons, the frequency of variants catalogued in disease specific mutation databases was at least 10 times higher than expected based on the population incidence of these disorders (Refsgaard, Holst et al. 2012; Andreasen, Nielsen et al. 2013; Risgaard, Jabbari et al. 2013). One explanation is that these databases were created many years ago, when we lacked the recent insight in the vast amount of normal human genetic variation. At that time, only common variants were known. Variants representing over 1 % of alleles were considered to be non-functional and called polymorphism. Variants with a lower frequency were (falsely) assumed to be functional. Thus, when a novel variant was detected in a gene causing the disorder in a patient, it was considered to be a pathogenic mutation when it was not observed in 100 controls, and/or when it segregated with the disease in the (often small) family. It is now clear that there is an enormous amount of rare variation, mostly without functional consequences. Efforts to curate the existing databases are ongoing, complemented to the creation of novel databases such as ClinVar (Landrum, Lee et al. 2014).

One of the lacunae in our current knowledge is the capacity to predict functional consequences of variants. Variants that are likely to disrupt gene function include nonsense, frameshift and canonical splice site (+/- 1 or 2) variants, but one needs to be cautious (reviewed by Richards et al., 2015). The main interpretational challenge resides with non-synonymous SNVs. An increasing number of tools exist that predict deleteriousness for missense mutations. These are based on various sources of information, such as evolutionary conservation, the biochemical consequences of the change in amino acid, the domain in which they occur etc. The far from perfect concordance of the results of different tools, their low accuracy (65-80 %) and low specificity illustrate that they are not sufficiently reliable to be used in a routine clinical setting (Thusberg, Olatubosun et al. 2011). However, one can expect that the increased knowledge and the use of combinations of different tools will lead to more reliable results (Dong, Wei et al. 2015).

In severe phenotypes, de novo mutations are the most common type of mutations (Gilissen, Hehir-Kwa et al. 2014; Wright, Fitzgerald et al. 2015). Therefore, a trio approach, which compares genomic sequences of parents and their affected child is a very efficient strategy to identify causal mutations. However, de novo on its own is not a sufficient criteria anymore to establish causality when using a genome-wide screen: each individual carries on average about 50 de novo SNV's in their genome, and this number increases with paternal age (Kong, Frigge et al. 2012). Of these, on average 1-2 are in the exome (coding or splicing regions (Wright, Fitzgerald et al. 2015). Also, a de novo CNV occurs in approximately 2-3 % of the normal population (Levy, Ronemus et al. 2011; Sanders, Ercan-Sencicek et al. 2011; Wright, Fitzgerald et al. 2015). This complicates the interpretation. For instance, in an exome sequencing study of individuals with autism, their parents and unaffected siblings, the same number of de novo SNV's was observed in cases and their unaffected siblings. However, the functional effects of these variants differed, including more de novo loss-of-function mutations in cases, as reviewed by Ronemus et al., 2014 (Ronemus, Iossifov et al. 2014). A related question is how to establish a causal relationship between a (mutated) gene and a specific certain phenotype. Since large cohorts of children with ID are being sequenced, the same gene could repeatedly be mutated merely by chance. Stringent criteria therefore need to be applied, incorporating gene specific mutation rates such as gene length (Wright, Fitzgerald et al. 2015). The de novo hypothesis does not apply for autosomal recessive conditions and should be interpreted with caution for X-linked recessive since the mother may be an asymptomatic carrier. For less severe autosomal dominant phenotypes, one should take into account the possibility of variable expression and reduced penetrance. Phenotyping of the parents and constructing a detailed pedigree therefore remains important.

Another level of variant interpretation concerns the phenotypic effects of the found variant. For most genes with exception for haploinsufficient genes, loss-of-function will only result in a phenotype when both alleles are affected. On the X-chromosome loss of function of over 1 % of X-linked genes is compatible with normal life in males, a truly unexpected finding (Tarpey, Smith et al. 2009). Dominant effects may result from haploinsufficiency, whereas for other genes, a dominant phenotype may result only when the protein is altered in such a way that a novel dominant negative or gain-of-function effect results.

Ultimately, existing knowledge on genotype-phenotype associations will be the final guide to decide which variants in which genes are disease causing. Building and curating databases containing both clinical and genotypic data requires continuous international efforts. DECIPHER, (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) is the main example of a successful initiative, which has contributed significantly to the identification of novel causal CNV's, and performs a key role as a reference for the clinical interpretation of array-CGH in diagnostic laboratories. Accurate phenotypic data remain however crucial.

In many instances, a variant (be it CNV or SNV) cannot unambiguously be classified as pathogenic versus non-functional. For this reason, variants are classified according to their likelihood of being causal using a five-tier system (ranging from known pathogenic to known non-functional).

Another complicating factor in the introduction of genome-wide screening is the potential of incidental findings, i.e. variants which have clinical significance, but are not related to the primary question. Whereas this might be perceived as a threat, many see this as an opportunity and therefore advocate to actively search for such so-called secondary variants in a limited set of genes, considered to be clinically actionable (Green, Berg et al. 2013; May 2015).

1.5 Studies on the aetiology of ID in Central Africa.

The prevalence of ID is higher in developing countries compared to the developed ones (Durkin 2002) and some ascribe this to various environmental and societal risk factors, many of which are interrelated. Examples include poverty, malnutrition, birth trauma, low parental education, high maternal age, high birth order, lack of stimulation, consanguinity and infectious diseases (Bashi 1977; Bergen 2008; Van Rie, Mupuala et al. 2008; Adnams 2010).

To date, etiological studies of ID in Central Africa are almost non-existent. The situation in developing countries stands in sharp contrast to the tremendous progress in genetic knowledge and technology

in the developed world. These countries face not only an almost complete lack of access to diagnostic technologies, but also lack of up-to-date scientific knowledge and training in genetics. As a result, mystical and traditional beliefs on the causes of ID often prevail, further hampering scientific progress (Kromberg, Zwane et al. 2008; Njenga 2008). In a survey of mothers of disabled persons in a special institution in Nigeria, Abasiubong et al. (Abasiubong, Obembe et al. 2008) found that only 10 % of mothers linked the disability of their child to biological factors whereas the vast majority of mothers incriminated religious and mystical factors. The impact of raising a child with ID is very high for a mother, but cultural attitudes and beliefs surrounding disability - which are largely negative - contribute greatly to mothers' disempowerment in the developing world (Abasiubong, Obembe et al. 2006). There is a paucity of research publications on mental health in DR Congo. The traditional and most common belief is that a diseases in general are either curses or a punishments. Curses are imposed by evil spirits or witches whereas the punishments are inflected by "Nzambi ya Mpungu" (Almighty god) or unhappy ancestors in reaction to misconducts. Reporting about mental health in DRC Congo, the CDC (Centres for Disease Control, USA) stated that "Mental illness is often considered a curse (sometimes believed to be caused by supernatural elements or the result of witchcraft)". (http://www.cdc.gov/immigrantrefugeehealth/profiles/congolese/background/index.html).

In 2010, the UNICEF reported a conception that is getting more and more popular in some African countries, including the DRC, that children with a mental disability are themselves accused of being "child witches" (UN April 2010, 2, 40).

As in majority of developing countries, the care for individuals with ID is poorly organized in the DR Congo. Recently, there has been an increased interest for mental health in the DR Congo. This was initiated by the implementation of a National Program for Mental Health, under the umbrella of Public Health Ministry. Following this initiative, the international NGO Handicap International implemented a network of institutions to provide care for people with special needs. However, these efforts are still insufficient. As an illustration, there are only 13 institutions specialized in mental health for more than 8 million inhabitants in Kinshasa. In Katanga, the second largest province of DR Congo, there is only one specialized institution. Moreover, almost all these institutions are private and registration is subjected to payment of expensive tuition fees. Since most families have limited financial means, the overwhelming majority of children with ID do not attend a school and do not receive any remediation. In addition, many of the existing institutions are not engaging in research on ID.

Research into the genetic causes of ID in a developing country can break the vicious circle. It will provide sound scientific data on genetic factors in the aetiology of ID and incidence of specific genetic

disorders. Reaching an etiological diagnosis in a person with intellectual disability is crucial for health care management in the society. The identification of preventable causes such as infections, congenital hypothyroidism or malnutrition is essential for accurate prevention programs. Also, on an individual level, an exact etiological diagnosis provides the parents and caregivers with a more accurate prognosis. It improves management and allows counselling on recurrence risks (Moeschler and Shevell 2014). In addition, this also offers the opportunity to openly discuss false believes and feelings of guilt.

However, the challenges for genetic studies of ID are huge. From a clinical point of view, knowledge on specific genetic disorders or syndromes is based on Caucasians. The reliability of these clinical criteria for specific syndromes established in Caucasians has not been investigated in central Africa. For instance, pictures of patients with African ancestry are almost completely lacking in the illustrated series of Terms in Morphology (Allanson, Cunniff et al. 2009). Environmental and societal risk factors for ID are more prevalent in developing countries. Thus, one can expect more complex causes of ID. State-of-the art genetic laboratory testing offers tremendous possibilities in reaching a diagnostic. However, the technical challenges in Central Africa are enormous, with a lack of laboratory infrastructure for molecular techniques. In addition, the lack of genetic studies in local populations results in the absence of reliable reference genetic data (e.g. CNV's of SNV's), which hampers the interpretation of genetic data, e.g. when deciding if a variant is causal. To illustrate this, the incidence of secondary variants detected in large scale exome studies in African-Americans is about half of that of Caucasians, indicating that the genetic databases are not representative for this part of the population (Dorschner, Amendola et al. 2013; Amendola, Dorschner et al. 2015). Finally, the mystical believes may also deter parents from participating in such studies.

On the other hand, there are also unique opportunities for research. DR Congo is a "mosaic" country with more than 220 ethnicities, which are groups of clans or people sharing the same language, history and traditions. In addition, people within an ethnic group are characterized by close morphological appearance. These are organized in 4 main ethnic groups including Bantous, Sudanese, Nilotic and Pigmies. The Bantous ethnic group constitute about 80 % of the Congolese population. The province of Kinshasa, where the current study was conducted, is located in the West of DR Congo. The population of Kinshasa is a melting-pot of people from various groups. However, majority of the population is from the Bantous group and migrated from neighbouring west, north and centre provinces. There are more than 220 local dialects and 4 official national languages in DR Congo. Three of the 4 are of Bantous in origin. All genetic studies indicate a large genetic variation of the African population. Previous studies have shown that the Bantous group has undergone multiple interactions with surrounding groups at different locations, thus resulting also in a large genetic diversity within

the Bantous (Marks, Montinaro, et al., 2015). Noticeably, with exception of the pigmy population, Congolese have not been included in earlier genetic studies, and details about genetic diversity in the Congolese population groups are still lacking.

However, based on the wide ethnic diversity, the presence of large families and frequent consanguinity in certain ethnicities, the DR Congo offers unique opportunities for genetic studies on the aetiology and mechanisms of ID (Gurdasani, Carstensen et al. 2015).

Research in ID in the DR Congo has the additional perspective of building capacity in the disciplines of medical genetics, (paediatric) neurology and psychiatry. There is a need for medical doctors who are able to think and act scientifically when confronted with ID, i.e. able to recognize, categorize, and identify possible etiological factors, select adequate diagnostic steps, counsel the parents and to perform research in this field. The present research project is one step towards reaching this goal.

Full references for articles cited in the general introduction can be found after the general discussion.

AIMS OF THE STUDY

A first aim of this study is to contribute to the understanding of the genetic aetiology of ID in Central Africa. We will therefore

- identify the aetiology of ID in a cohort of Congolese individuals with unexplained ID, using state-of-the art clinical and genetic studies.
- define the incidence of Fragile-X syndrome in this cohort and evaluate the usefulness of three different checklists as a screening tool.
- define clinical parameters associated with a higher chance of reaching an etiological diagnosis.

A second aim is to gain insight into the variability in clinical expression of specific genetic disorders in Central Africa. This will be done by comparison of phenotypic manifestations in Congolese cases with those in the literature.

The third aim is to gain insight into causes of and variability in genetic mutations observed in Congolese patients with a genetic disorder, through the study of cases we identify.

A CLINICAL AND GENETIC STUDY IN 127 PATIENTS WITH ID IN KINSHASA, DR CONGO

(Manuscript in preparation)

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1.6 Introduction.

Reaching an etiological diagnosis often has a major impact on the management plan for a child with ID or a developmental disorder. Not only can this guide towards efficient care, but also it will make possible the implementation of preventive measures such as pre-conceptual counselling and prenatal testing. In the African context, were ID is mainly attributed to mystical causes (Abasiubong, Obembe et al. 2008; Njenga 2008), a study of genetic causes will provide strong arguments to campaign against false beliefs and help to improve actions of caregivers. Data on the genetic aetiology of ID in Central Africa are scarce. With exception for some case reports, only few studies have investigated a cohort of patients with ID using advanced technologies (Uwineza, Caberg et al. 2014).

From a clinical point of view, clues towards a possible diagnosis can be retrieved from the family history, the personal medical history as well as from the development and behaviour. In the clinical examination, special attention is given to the presence of dysmorphism, biometry (especially head circumference) and neurological findings. When a clinical diagnosis is suspected, targeted (genetic) testing can be undertaken. For certain clinically recognizable conditions (e.g. VATER association), no cause is known and the diagnosis remains purely clinical. When no clinical diagnosis can be made, genetic screening can be performed. According to a consensus statement, genome-wide screening for chromosomal imbalances is considered to be a first-tier test, replacing the traditional karyotyping (Battaglia, Doccini et al. 2013; Moeschler and Shevell 2014).

Genetic causes have been detected in 4 to 41 %, depending of the recruitment strategy, type and resolution of techniques (Battaglia, Bianchini et al. 1999; Moeschler 2008; Bernardini, Alesi et al. 2010). Reaching a genetic diagnosis in an individual with ID is based on different but complementary approaches. Chromosomal aberrations represent the major diagnostic category in the older studies (Devriendt, Holvoet et al. 2003; Rauch, Hoyer et al. 2006). Depending on the resolution of the applied platform, submicroscopic imbalances represent 6 to 20 % of causes (Rauch, Hoyer et al. 2006; Chong, Lo et al. 2014; Nicholl, Waters et al. 2014; Utine, Haliloglu et al. 2014). The most common monogenic cause of ID is the Fragile-X syndrome, that results from mutations in the *FMR1* gene (MIM 309550). Since the vast majority of cases are caused by an expansion of a CGG repeat in the 5'-UTR of the gene, a straightforward genetic screening test exists assessing the number of CGG repeats. For this reason, all patients with ID are investigated for the CGG repeat size (Moeschler and Shevell 2014). Mutations in X-linked genes are a common cause of ID and explain an excess of males with ID (Mandel and Chelly 2004; Ropers 2010). X-linked ID is observed in 6-12 % of males with ID (Hu, Haas et al. 2015; Tzschach, Grasshoff et al. 2015). However, XLID is heterogeneous with more than 100 genes identified to date (Hu, Haas et al. 2015). Thus NGS offers opportunity to screen for x-linked ID. X-linked inheritance is

suspected when a clinically recognizable X-linked syndrome is observed or when ID segregates in the pedigree in a clear X-linked way. In addition, unaffected mothers who are carrier of certain X-linked mutations have a skewed X-inactivation pattern, meaning that a preferential inactivation mostly occurs of the X-chromosome that carries the mutation. An example is the ATRX-syndrome. Thus, skewed X-inactivation in a mother of a boy with unexplained ID may point to an X-linked cause. Plenge et al. (2002) studied X-linked ID families, and demonstrated a high incidence of skewed X-inactivation in carrier females (Plenge, Stevenson et al. 2002). Rauch et al. (2006) reported that 5.6 % of mothers of boys with unexplained ID have more skewed of X-inactivation (> 95 %) suggesting X-linked ID (Rauch, Hoyer et al. 2006). Also, females may present X-linked disorders, typically because the dominant effect of a mutation. This is seen in incontinentia pigmenti caused by mutations in the IKBKG or NEMO gene, and in females with a CASK mutation (Martinez-Pomar, Munoz-Saa et al. 2005). Also, skewed X-inactivation, with preferential inactivation of the normal allele may result in an X-linked condition in a female (Esquilin, Takemoto et al. 2012)

Whole Exome sequencing (WES) is an emerging technology and a powerful means to identify a causal SNV in autosomal genes. However, one of the limiting factors is the large number of unclassified SNV's each individual carries. Therefore, additional filtering based on the phenotype, supposed inheritance pattern (e.g. X-linked ID or autosomal recessive ID) and population specific polymorphisms aids in further limiting the number of potential candidate causal SNV's. In this study, we took two different approaches. First, we selected cases with a high probability of X-linked ID. In the absence of clearly X-linked pedigrees, we took skewed X-inactivation in the mother of a boy with unexplained ID as an indication of possible XL-ID, as discussed above. Likewise, skewed X-inactivation in a girl with unexplained ID may also indicate that an underlying X-linked mutation explained her ID. Therefore, we undertook an X-inactivation screening of all available mothers of boys in our cohort, as well as all female patients. Next, in two sisters with syndromic ID, an autosomal recessive cause was thought to be the most likely inheritance pattern. We performed exome sequencing in both of them and filtered for shared variants. In addition, under the hypothesis of a de novo dominant mutation and germline mosaicism in one of the parents, we also filtered for potentially pathogenic variants shared by the two sisters in the genes causing AD ID (based on the DDD2G gene list).

These general principles form the basis of the outline of our study, as illustrated in figure 1.

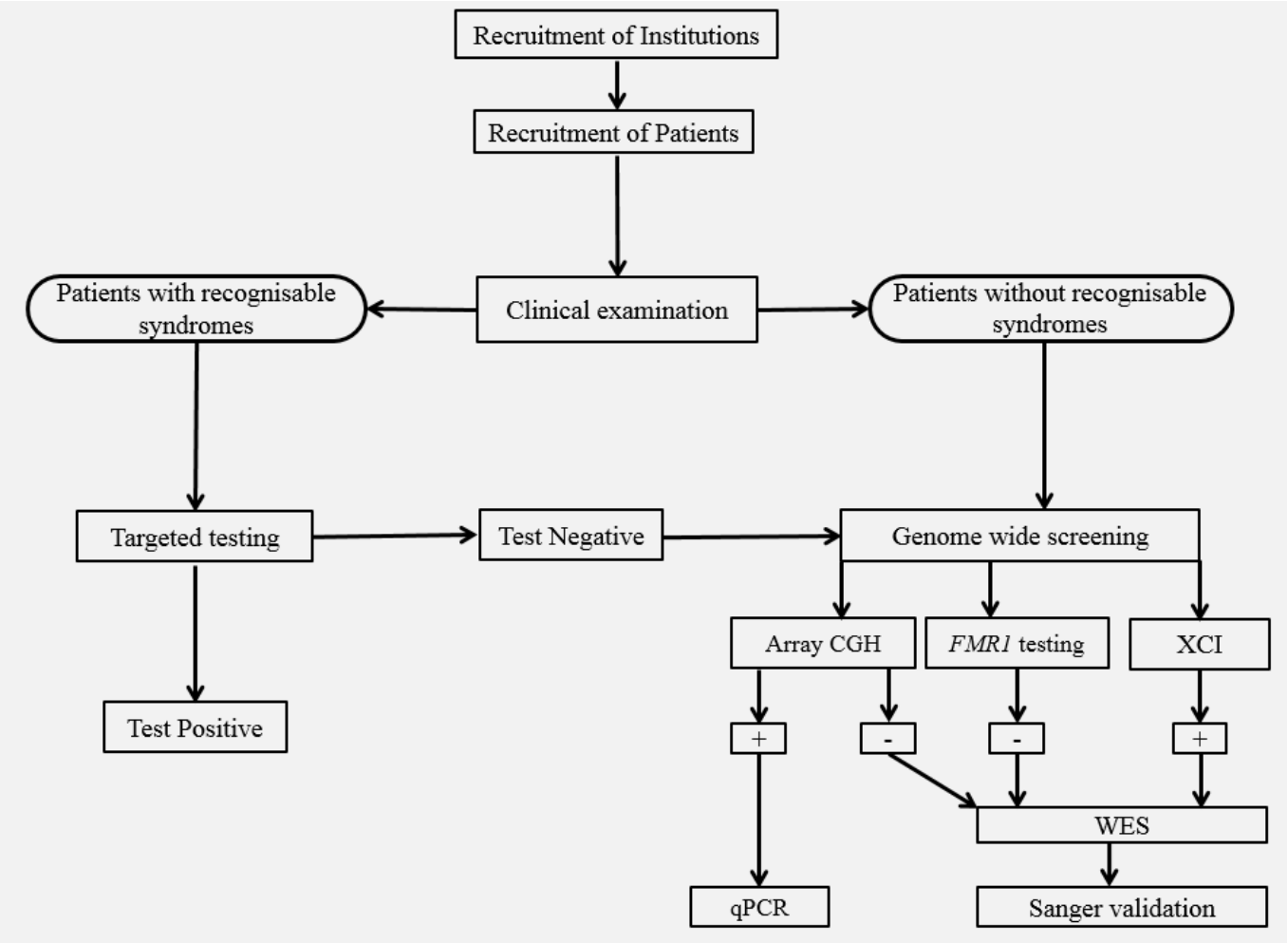

Figure 1. Flow chart of the current study.

Clinical examination was at the centre of the current study. Depending on the out-come, either a targeted testing or a wide genetic screening was decided

1.7 Materials and methods

1.7.1 Description of the recruitment of the cohort

In 2009 the International Non-Governmental Organization Handicap International identified 13 institutions specialized for intellectual disability in Kinshasa. We contacted the directors of these institutions, explained our project and asked whether they were willing to collaborate and allow us to recruit patients from their institutions to participate in the study. Two hospitals and 4 specialized schools agreed to participate: University Hospitals of University of Kinshasa (CUK); Centre de Neuro-PsychoPathologie de Kinshasa (CNPP); Village Bondeko Kabambare; Centre d'Etude, d'Intervention et d'Evaluation pour le Handicap Mental et l'Autisme (CEIEHMA); Centre Pour Enfant Bon Départ (CPEBD) and Ecole primaire Notre Dame d'Afrique. We included patients with intellectual disability (ID) after obtaining informed consent from their parent(s) or legal representative(s).

In each of these 6 institutions we worked in close collaboration with the directors and applied a stepwise recruitment and consent process:

1° Contact by mail. The institution provided the parents an envelope containing the leaflet explaining the study, a copy of the study questionnaire and an invitation to attend an information day at the institution. We wished to inform the parents of our study, prepare them to our questions and allow them to consider specific questions they may wish to ask us during the information day scheduled 1 week later.

2° Information day: during this meeting, we introduced ourselves and provided a short overview on ID, genetics and genetics of ID. We discussed in details the aims of the study, the methods and foreseen issues, what was expected from them and what they could expect from us. We took questions from the attendants during a plenary session. This was followed, when necessary, by a private conversation in a separate room. At the end of the meeting, we invited parents who wished to participate to sign and return the consent form, after an additional week of reflection. These children were then examined at a later date in the institution.

3° The clinical examination was done at a later occasion, at the institution. We conducted a standardized clinical examination including body measurements and a dysmorphological evaluation. Personal and family history was obtained from the parents and tutors. Body measurements were converted into standard deviation scores using the Centres for Disease Control (CDC, USA) growth charts. We used the obtained information to score three fragile-X screening checklists (Hagerman,

Amiri et al. 1991; Maes, Fryns et al. 2000; Guruju, Lavanya et al. 2009). Familial ID was defined as a family history with another ID patient (up to 3rd degree relative). Otherwise, the patient was classified as sporadic ID. A patient was classified as dysmorphic when he had either 2 major malformations or ≥ 3 minor anomalies (Hennekam 2011). We referred to the Elements of Morphology: Standard Terminology published in 2009 (Allanson, Cunniff et al. 2009; Biesecker, Aase et al. 2009; Carey, Cohen et al. 2009; Hall, Graham et al. 2009; Hennekam, Cormier-Daire et al. 2009; Hunter, Frias et al. 2009; Klinger and Merlob 2009).

We finally included, collected clinical information and obtained DNA from 127 patients (33 females and 94 males), aged from 1.24 to 24.65 years and with a mean of 10.03 ± 4.68 years.

1.7.2 Technology:

1.7.2.1 DNA extraction

Venous blood was obtain from a peripheral vein and genomic DNA was extracted by the salt saturation method as previously described (Miller, Dykes et al. 1988). Between 2010 and 2012, blood samples or leucocytes pellets were shipped to Belgium by airplane and DNA extraction was done in the Centre for Human Genetic in Leuven. In meantime we managed to establish a reliable DNA extraction facility at the Institut National de Recherche Biomédicale (INRB) in Kinshasa. From 2013 on, the DNA extraction facility at the INRB was fully operational and we were able to perform DNA extraction in Kinshasa and ship the extracted DNA to Belgium for further analyses.

1.7.2.2 FMR1 testing

To assess the fragment size of the CGG repeat in the promoter region of the FMR1 gene, the target region was amplified during a PRC reaction using the PRC-enhancer kit (Invitrogen) and the FRAXA-A and FRAXA-B primers. The reaction mix contained 5.6 µl of water, 2 ml of the 10X PCRx Amplification buffer, 0.6 µm of 50 mM MgSO4, 1 µl of dNTPs (4 mM), 8 µl of PCRx Enhancer Solution, 1.5 µl of Primer mix (10 pm/µl) and 0.25 µl of Taq DNA Polymerase from Roche. The cycling comprised an initial denaturation at 95°C for 3 minutes followed by 27 amplification cycles made of short denaturation at 95°C for 15 seconds, annealing at 64°C for 1 minute and elongation at 75°C for 1 minute. The reaction was terminated with a final elongation at 75°C for 7 minutes and cooling at 15°C ∞. PCR control was done on 2 % agarose gel with 1 kb size marker. The PCR product was resuspended with a mixture of 20 µl HiDi Formamide (Applied Biosystems) and Rox 500 (Applied Biosystems). Fragment were separated on the on ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA 94404 USA) then analysed with GeneMapper Software.

1.7.2.3 Microarray-CGH using stripped slides

We performed whole genome copy number screening using CytoSure™ ISCA v2 array 8x60k format (OGT, Oxford UK). To make this technology accessible in a resource poor setting, we adapted the existing protocol to recycle arrays used once before. Used arrays were provided by the Cytogenetic laboratory, UZ Leuven. We validated the protocol using 9 DNA samples with previously characterized CNV's using high resolution Illumina SNP array v2.1 and CytoSure™ ISCA v2 array 4x180k format from OGT. These 8x60k slides have 8 slots and each slot contains 60.000 oligonucleotide probes. The backbone coverage is 1 probe every 60 kb but at each of the 480 targeted regions, the coverage is increased to 1 probe every 31 kb, corresponding on mean to 4 probes per gene in the targeted regions.

The validation experiment was done as follows:

a) We tested 3 slides that were used once within 7 days maximum. During their first use, these slides were labelled for 2 hours (at 37°C), hybridization for 16 hours, washed and scanned following the manufacturer's protocol then kept in a dehumidified cabinet after scanning.
b) We stripped the slides in the Little dipper robot, containing wash buffer 2 (350 ml) + MiliQ water (350 ml) in the dish 1 and 700 ml MiliQ water in the dish 2. The liquid in dish 1 was heated beforehand to 65°C at least 30 minutes beforehand and the stirring bar was set at speed 7. The liquid in dish 2 remained at room temperature. Used slides were washed for 30 minutes and submerged 20 times/minute. Slides were immediately transferred to the dish 2 were they were similarly submerged for 1 minute. Slides were dried at room temperature for 2 minutes then stored in a lightproof box in the dehumidified cabinet until their use.
c) For the re-hybridization, we used 9 samples (3 females and 6 males) from a previously studied autism cohort, fully characterized with CytoSure™ ISCA v2 array 4x180K and Illumina SNP arrays v.2. We run and analysed the female and male samples separately in groups of three patients, using the loop-design (Allemeersch et al., 2009.) The hybridization protocol was according to the manufacturer's except the following change: labelling at 37°C for 22 hours.

The protocol for patients' testing included:

a) Labelling, hybridization and washing were done following manufacturer instructions, with the exception that we obtained optimal dye incorporation when we extended the labelling duration to 22 hours in a lightproof box placed in a 37°C oven.
b) Arrays were scanned with Surescan High-Resolution Technology Microarray at 2 µm wave length, followed by feature extraction using Agilent Feature Extraction Software® v.10.10.11. Aberration detection was done by Circular Binary Segmentation (CBS) algorithm via CytoSure Interpret Software ® v.4.0. A CNV was reported as deletion when the Log2 ratio was < to -0.36 and duplication when > to +0.36. Genomic coordinates were based on the UCSC February 2009 (hg19) (NCBI build 37). We used a loop strategy to compare sample to reference (Allemeersch, Van Vooren et al. 2009).
c) Data interpretation. We uploaded our data to Cartagenia CNV bench (Horpaopan, Spier et al. 2015). This allowed a more efficient classification of found copy number variations by comparison with databases of normal variation (Database of Genomic Variants) and pathogenic CNV's (DECIPHER, ISCA consortium) and known local pathogenic or benign CNV's.

1.7.2.4 q-PCR

To confirm microarray-CGH results, evaluate parental inheritance or confirm a clinical diagnosis of Down syndrome, we performed qPCR using the LightCycler480® (Roche). The Primer3web version 4.0.0 tool (http://bioinfo.ut.ee/primer3/ (last accession on 08 February 2015) was used to design primers listed at the end of the chapter. We used the ddCt relative quantification method (Sequence Detection System bulletin 2, Applied Biosystems) with SYBR-green. For Down syndrome, we used *NCAM2* (NM_004540) located on 21q21.1 as the target and the *SCN2A* (NM_021007) located on 2q24.3 as reference gene. Since the 2 primer sets showed similar efficiency, we were able to use the ddCt relative quantification method. The standard curve experiments were done with fourfold dilutions of genomic DNA, starting with 10 ng in the first dilution. The 20 µl reaction mixtures consisted of 10 µl of LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), 3 ml of PCR-grade water (Roche) 500 nM of each primer and 10 ng DNA. After an initial denaturation step for 10 min at 95°C, thermal cycling conditions were 10 s at 95°C and 30 s at 60°C for 45 cycles. When testing patients' samples, we included No-DNA templates and 3 calibrators (1 from DR Congo and 2 from Flanders) and all samples were run in Duplo following the standard 20 µl qPCR protocol.

1.7.2.5 X-chromosome inactivation pattern

The X-chromosome inactivation pattern was analysed through the polymorphic CAG-repeat in the androgen receptor gene using *Hpa*II restriction enzyme as described. We included *Rs*aI in the non-digested samples as the control enzyme. The proportion of X-inactivation of the two X chromosomes was calculated as follows: the proportion of digested alleles (A) was obtained by dividing the Area Under the Curve (AUC) of allele 1 by AUC of allele 2 after *Hpa*II digestion, whereas the proportion of non-digested alleles (B) was the ratio of AUC of allele 1 to AUC of allele 2 in the experiment using *Rsa*I digestion. The X-inactivation pattern was calculated with the following formula:

$\% = \frac{Rx100}{R+1}$ R being equal to $\frac{A}{B}$

1.7.2.6 Whole Exome analysis

Exome sequencing was done either in the Genomics core KULeuven (n=2) or in collaboration with the Medical Genetics Laboratories, Baylor College of Medicine (n = 10), using the SeqCap EZ Human Exome Library v3.0 (Roche, NimbleGen), and the Illumina HiSeq2000 platform. Data analysis was done using commercial and in-house developed software. Exome sequences were obtained from the patients only.

The variant filtering was done as described in table 1. We first filtered the variants under the hypothesis of an X-linked condition, followed by a possible autosomal dominant (AD) or autosomal recessive (AR) cause. During initial variant filtering we retained only non-synonymous variants in coding regions, including exonic and splicing variants (including the first 5 intronic nt). Next, we removed common variants using a cut-off of > 1 % for X-linked and AD or >2 % for AR in the 1000 Genomes Project and ESP databases. We further discarded variants observed in other Belgian and Congolese cases. We retained variant within genes on list of genes known to be implicated in developmental disorders and ID, obtained from the UK DDD2G project (Deciphering Developmental Delay), update of July 18th 2014. In addition, variants were further prioritized using three different *in silico* prediction tools (PolyPhen2, Mutation Taster or SIFT). Promising variants were validated with Sanger sequencing and inheritance was investigated when parental DNA samples were available.

Table 1. Variant filtering strategies

Steps	X-linked	Autosomal Dominant	Autosomal Recessive	
			Compound heterozygote	Homozygous
1	Retain coding variants	Retain coding variants	Retain coding variants	Retain coding variants
2	Filter out synonymous variants	Filter out synonymous variants	Filter out synonymous variants	Filter out synonymous variants
3	Filter out variants with frequency >1% in 1000 GP and ESP	Filter out variants with frequency >1% in 1000 GP and ESP	Filter out variants with frequency >2% in 1000 GP and ESP	Filter out variants with frequency >2% in 1000 GP and ESP
4	retain X-chromosome variants	retain heterozygous variants	retain heterozygous variants	retain only homozygous variants
5	Remove duplicates	Remove duplicates	Filter out variants reported as heterozygous in more than 5 or homozygous in one or more local controls (patients without ID)	REMOVE duplicates
6	Retain only SNVs heterozygous in female patient	Filter out variants observed in local controls (patients without ID)	Remove duplicates	Filter out variants reported as heterozygous in more than 5 or homozygous in one or more local controls (patients without ID)
7	Filter out variants observed in local controls (patients without ID)	Keep variants in genes from the DDD2G list	Filter out genes with only 1 SNV	Keep variants in genes from the DDD2G list
8	Keep variants in genes from the DDD2G list and look for concordance of phenotype	Keep deleterious SNVs s or missense SNVs predicted to be Disease Causing by at least 2 prediction tools	Keep variants in genes from the DDD2G list	Keep variants in genes involved in AR disorders and evaluate concordance of phenotype
9	Select variants in all X-linked genes - deleterious mutations (Splice, frameshift or stop) - missense variants predicted to be Disease Causing by at least 2 prediction tools (PolyPhen2, Mutation Taster or SIFT) and evaluate concordance of phenotype	Keep variants in genes involved in AD disorders and evaluate concordance of phenotype	Keep variants in genes involved in AR disorders and evaluate concordance of phenotype	

1.7.2.7 Sanger sequencing for Exome validation

Sequencing primers used for Sanger validation are listed at the end on the chapter. The 50 µl amplification reaction contained 5 µl of DNA (50 ng), 5 µl of PCR reaction buffer + Mg2+ (Roche), 5 µl of each Primer solution (2.5 pmol/µl), 5 µl of dNTP's (2 mM), 0.5 µl (2.5 U) of Taq DNA Polymerase (Roche) and 24.5 µl of Ultrapure water (Baxter). The PCR was run on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA 94404 USA) using the following program: initial denaturation stage at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 45 s; and a final extension stage at 72 °C for 5 min. PCR products were sequenced using Big Dye termination method and detected with ABI 3730xl DNA Analyzer instrument (Applied Biosystems, Foster City, CA 94404 USA).

1.7.2.8 Fragment analysis for ARX CAG repeat

We used 2 primer sets (Primer 4044 ARX-2F and JR2-FAM) to amplify the first 2 CAG stretches in the ARX. The reaction mix contained 1 µl of DNA, 5 µl of 10X amplification buffer, 5 µl of dNTP's (2 mM), 15 µl of 3X PCR enhancer, 1.5 µl of 50 mM MgSO4, 12 µl of water and 0.5 µl of Taq DNA Pol. (5U/µl). The 35 cycles had the following conditions: 95°C for 30 sec, 58°C for 30 sec and 68°C for 60 sec. 0.1 µl of PCR product was re-suspended in 20 µl HiDi Formamide/Rox500 and loaded onto a Genetic Analyzer for Fragment separation. The separated file was analyzed with GeneMapper® v.5.

1.7.3 Ethics

The participants were informed about the structure and aims of the study. They were informed concerning their right to withdraw from the study. For each participant, parents or legal representatives provided written consent for study participation. We applied an anonymous and non-personal coding system to protect participants' privacy. Our research protocol was approved under the number ESP/CE/008/2015 by the National Ethical Committee of the Public Health School of the University of Kinshasa, Kinshasa, and the DR Congo.

1.7.4 Statistics

In order to conclude on the QC-Metric from the striped slides, we used the statistic tool R to compute the Paired Wilcoxon Ranked Sum Test P-Value.

We used WinPePi v.11 to build confidence Intervals for the proportions of XCI, at a 95 % confidence.

1.8 Results

1.8.1 General clinical characterization of the cohort

A total of 131 patients were initially recruited for the study from 6 institutions in Kinshasa in the Democratic Republic of Congo. Four were lost to follow-up, thus this study will report on 127 patients. Figure 2.A presents the origin of recruitment of the 127 remaining participants. There were 33 (25.98 %) girls and 94 (74.02 %) boys (Figure 2.B), with an age 10.32 ± 4.68 years (ranges 1.24 - 24.65). Only 7 patients were aged equal or above 18 years, 120 were below 18 years.

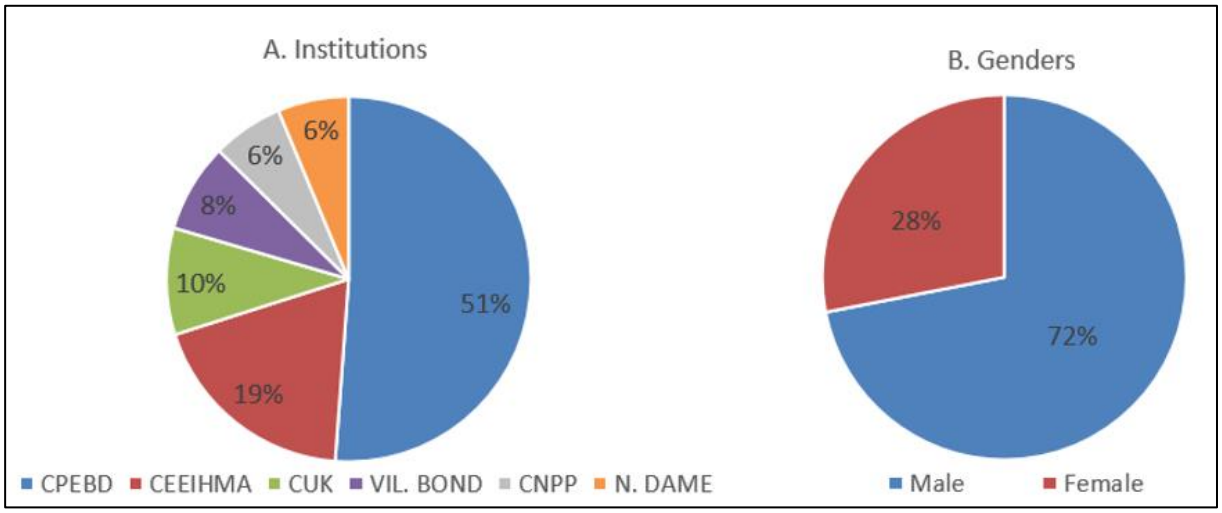

Fig 2. Panel A. Distribution of patients based on Institution of origin. CPEBD= Centre Pour Enfants Bon Départ; CEEIHMA= Centre d'Etude, d'Intervention et d'Evaluation du Handicap Mental et l'Autisme; CUK=Cliniques Universitaires de Kinshasa; Vil. Bond= Village Bondeko; N. Dame= primary school Notre Dame; CNPP= Centre de NeuroPsycho-Pathologie of Kinshasa. Panel B. Gender distribution, note unequal distribution.

The IQ levels were available for 72 (56.69 %) patients (figure 3) and had been determined by various intelligence tests, depending on the school or institution.

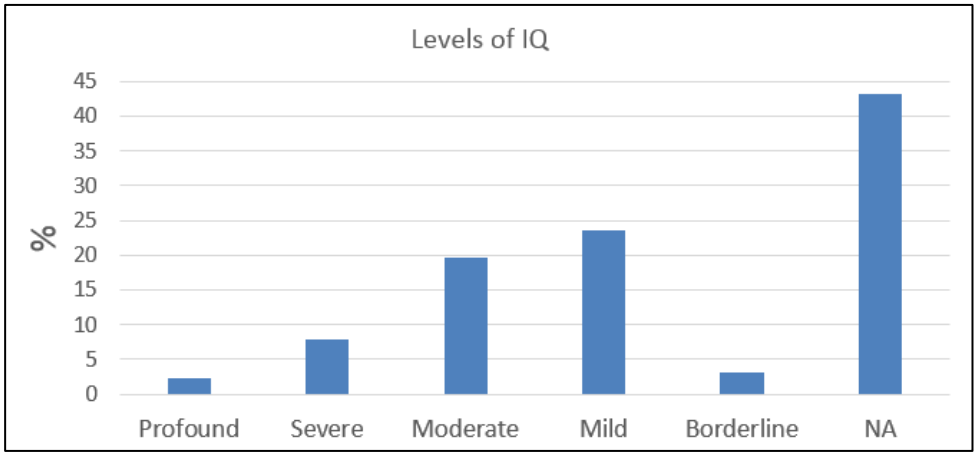

Figure 3. IQ level of participants in this study.

Family history was negative with regard to ID in 113 patients, 14 had a positive family history of ID (up to the third degree).

Personal history and clinical examination revealed neurological manifestations in 24 patients, including seizures in 15, deafness in 4, cerebral palsy in 3 and dystonia in 2. Nine patients had a major malformation at birth including congenital heart defect in 2, congenital inguinal hernia in 2 and one each of coloboma of eyelid, congenital cataract, cryptorchidism, limb reduction defect, micro-penis and a congenital testicular cyst. Microcephaly (OFC ≤ - 2 SD) was present in 38 patients and macrocephaly (OFC ≥ +2SD) in 10 (figure 4). The OFC was not available in 1 patient.

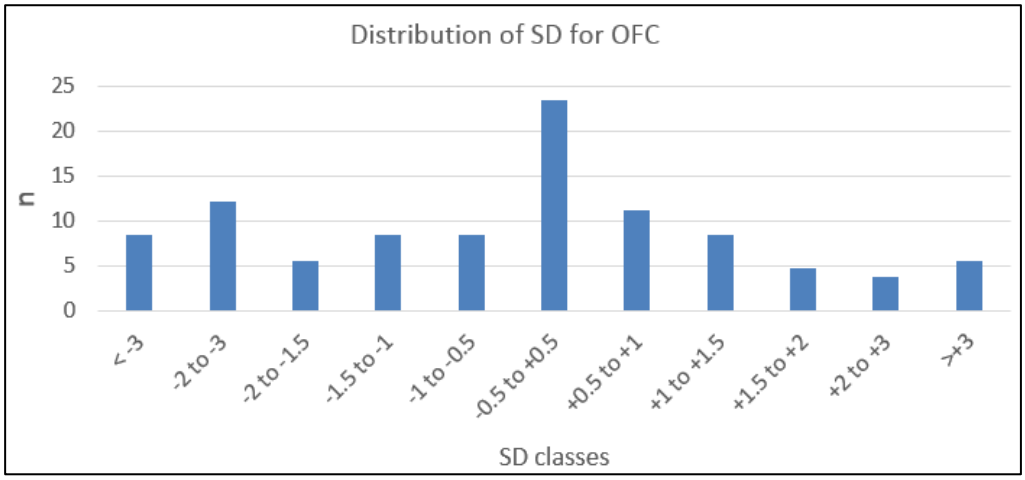

Figure 4. Distribution of head circumference.

The number of minor anomalies was recorded for each individual (Figure 5). Fifty (45.87 %) of the 108 patients (after exclusion of the 19 Down syndrome patients) had three or more minor anomalies, and were considered to be dysmorphic.

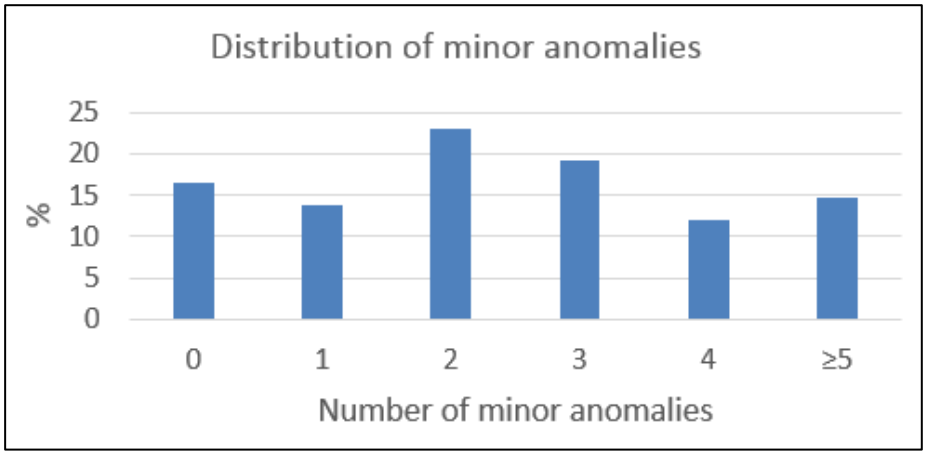

Figure 5. Distribution of the number of minor anomalies in patients (after exclusion of Down syndrome cases).

1.8.2 Clinically recognized syndromes.

In 19 patients, the diagnosis of Down syndrome was made. In addition to this, we clinically diagnosed one patient with Williams-Beuren syndrome (WBS), Partington syndrome, Noonan syndrome, Oculo-Auriculo-Vertebral Spectrum (OAVS) and Amniotic Bands Sequence. In 8 individuals, the most likely diagnosis was an acquired cause of their ID. Below, we describe and discuss the patients with a clinical diagnosis.

1.8.2.1 Down syndrome.

We identified features consistent with Down syndrome in 19 patients aged 1.86 to 17.08 years. Fourteen (73.68 %) were born to mothers aged above 35 years. Maternal age was not available for one. Microcephaly was present in 16 patients and was associated with respectively upslant of eyes and hypertelorism in 14, epicanthus and sandal gap in 13, respectively (Table 2). None of them had a diagnosed heart defect or another major congenital malformation.

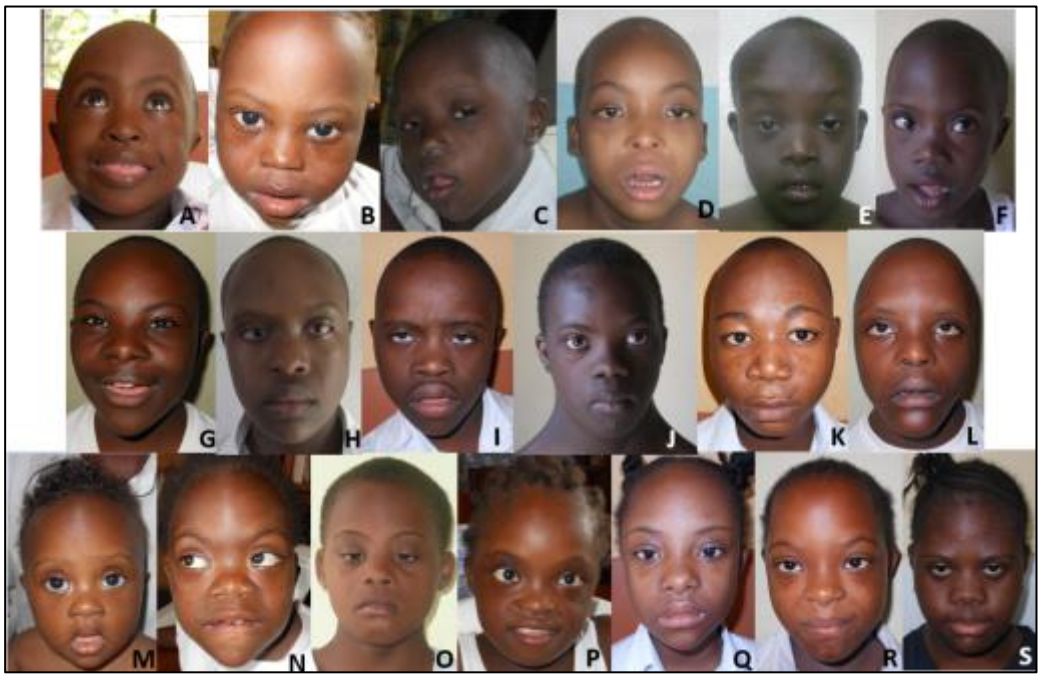

Figure 6. Faces of patients with Down syndrome. Patients sorted in accordance with table 2.

The diagnosis of Down syndrome was confirmed in all cases, using different techniques depending on the time of recruitment. In the first 6 clinically diagnosed DS patients, a G-banded karyotype was done. In a second group of 6 patients, microarray-CGH confirmed a duplication of the entire chromosome 21. Finally, in the remaining 7 patients, as well as the previous 12, the diagnosis was confirmed by means of q-PCR (see materials and methods section) (figure 7). In one patient (DRC-0157), with a clinical diagnosis of Down syndrome (figure 6-K, figure 7 and table 2), we observed a relative ratio of 1.2, consistent with mosaicism for trisomy 21.

Table 2. Description of patients with Down syndrome

ID	Gender	Age (Y)	Maternal age	OFC (z sc)	weigh (Z-sc)	Heigh (Z-sc)	Upslant of eyes (14/19)	Hyper-telorism (14/19)	Epicanthus (13/19)	Sandal gap (13/19)	Flat face (11/19)	Brachy-cephaly (10/19)	Transv erse palmar crease (10/19)	Small/ flat nose (7/19)	Bra-chydactyly of toes (7/19)	Tongue protrusion (6/19)	Low posterior hairline/ webbing (4/19)	Low set ears (3/19	Karyotype (6/19)	aCG (6/1	qPCR (rel. qant) (19/19)
DRC-0100	M	4,21	37,97	-2,06	0,70	-0,72	+	+		+	+		+			+		+	NA	T21	1,587401
DRC-0015	M	4,52	36,66	-3,6	NA	NA	+	+	+		+			+					NA	T21	1,52274
437634	M	5,08	NA	-2,74	-1,80	-0,91	+			+		+	+			+			T21	NA	1,543993
437587	M	6,88	40,25	-1,43	NA	NA		+		+			+					+	T21	NA	1,470867
447754	M	7,00	39,62	-0,42	NA	-2,77	+	+		+	+	+	+	+					T21	NA	1,491399
437573	M	7,70	39,41	-2,21	-1,64	-1,82	+	+	+		+			+		+			T21	NA	1,450617
DRC-0051	M	12,52	35,62	-2,2	0,11	-1,6	+	+	+	+	+	+	+		+		+		NA	NA	1,501773
437632	M	13,38	31,76	-3,07	NA	NA		+	+	+				+					T21	NA	1,570982
DRC-0161	M	13,98	36,79	-3,21	-2,39	-3,14			+	+	+	+			+	+	+		NA	NA	1,538652
437590	M	14,65	36,49	-2,7	NA	NA	+	+				+	+				+	+	T21	NA	1,522737
DRC-0157	M	17,03	26,15	-2,57	-1,89	-4,24	+	+	+			+	Deep creases		+				NA	T21	1,203025
DRC-0041	M	17,08	42,02	-2,58	<-3	-3,69		+	+		+		+		+				NA	NA	1,470867
DRC-0013	F	1,86	24,32	NA	NA	NA	+	+	+	+	+			+	+	+			NA	NA	1,506987
DRC-0177	F	8,32	37,71	-3,69	-1,73	-3,33		+	+		+		+	+					NA	T21	1,881218
DRC-0058	F	9,51	26,43	-3,95	-2,84	-3,58	+		+	+		+	+						NA	NA	1,538652
DRC-0152	F	9,93	40,47	-2,56	-2,88	-2,41	+	+	+	+	+	+		+	+	+	+		NA	T21	1,707241
DRC-0174	F	11,03	40,79	-2,8	-2,34	-2,4	+			+									NA	T21	1,649086
DRC-0062	F	12,73	42,92	-2,28	-1,89	-3,09	+	+	+	+		+	+						NA	NA	1,517469
DRC-0171	F	13,73	43,27	-2,6	0,93	-1,47	+		+	+	+	+			+				NA	NA	1,396356

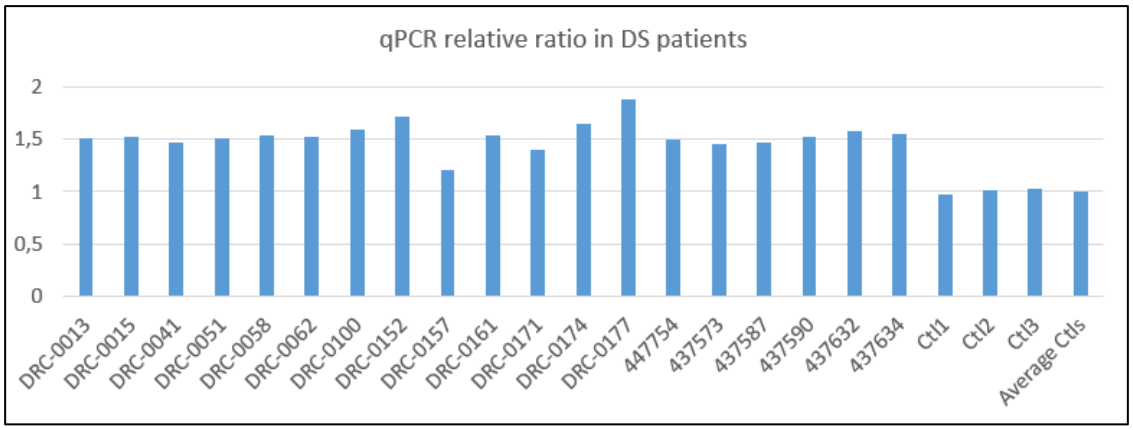

Figure 7. qPCR analysis confirming the diagnosis of trisomy 21 in all patients with the clinical diagnosis of Down syndrome.

1.8.2.2 Williams-Beuren syndrome.

The clinical features in the patient with WBS are described in detail in chapter 5.3 (Challenges for genetic studies in Africa). First, FISH testing was performed to confirm the suspected clinical diagnosis of Williams syndrome. Only one *ELN* signal was detected in all of the hundred cells analysed, whereas all cells had 2 signals for the control probe. Next, to define the exact size of the deletion, microarray-CGH was done using a stripped 8x60k slide. This revealed the recurrent 1.57 Mb deletion arr [hg19] 7q11.23(72,634,874-74,203,685)x1.

1.8.2.3 Partington syndrome.

This patient was 16.53-year-old born to unrelated parents and had normal functioning,. He was the second of 5 children including one older and 1 younger brother and 2 younger sisters (pedigree figure 8-A). Pregnancy and delivery were uneventful. He had mild ID and speech problems and went to a specialized primary school. He had a normal biometry (OFC 57.2 cm, 0.93SD; weight 61 cm, -0.19SD and height 172.5 cm, -0.28SD). He had a long and triangular face with large and protruding ears. He had marked dystonic movements of his hands and arms. Speech was dysarthric. He had mild ID. His older brother was equally affected and attended the same specialized school as the index. Clinically, he had a long and triangular face, large and protruding ears, scoliosis and the same dystonic movements and dysarthric speech as his brother (figure 8-B). The clinical diagnosis of Partington syndrome was made and subsequently confirmed by molecular testing of the Aristaless Related homeoboX (*ARX*) gene. Both brothers carry the recurrent duplication of 24 bp in exon 2 (c.429_452dup24), which results in an increase of a polyalanine tract in the ARX protein from 12 to 20 alanines (Curie et al., 2014).

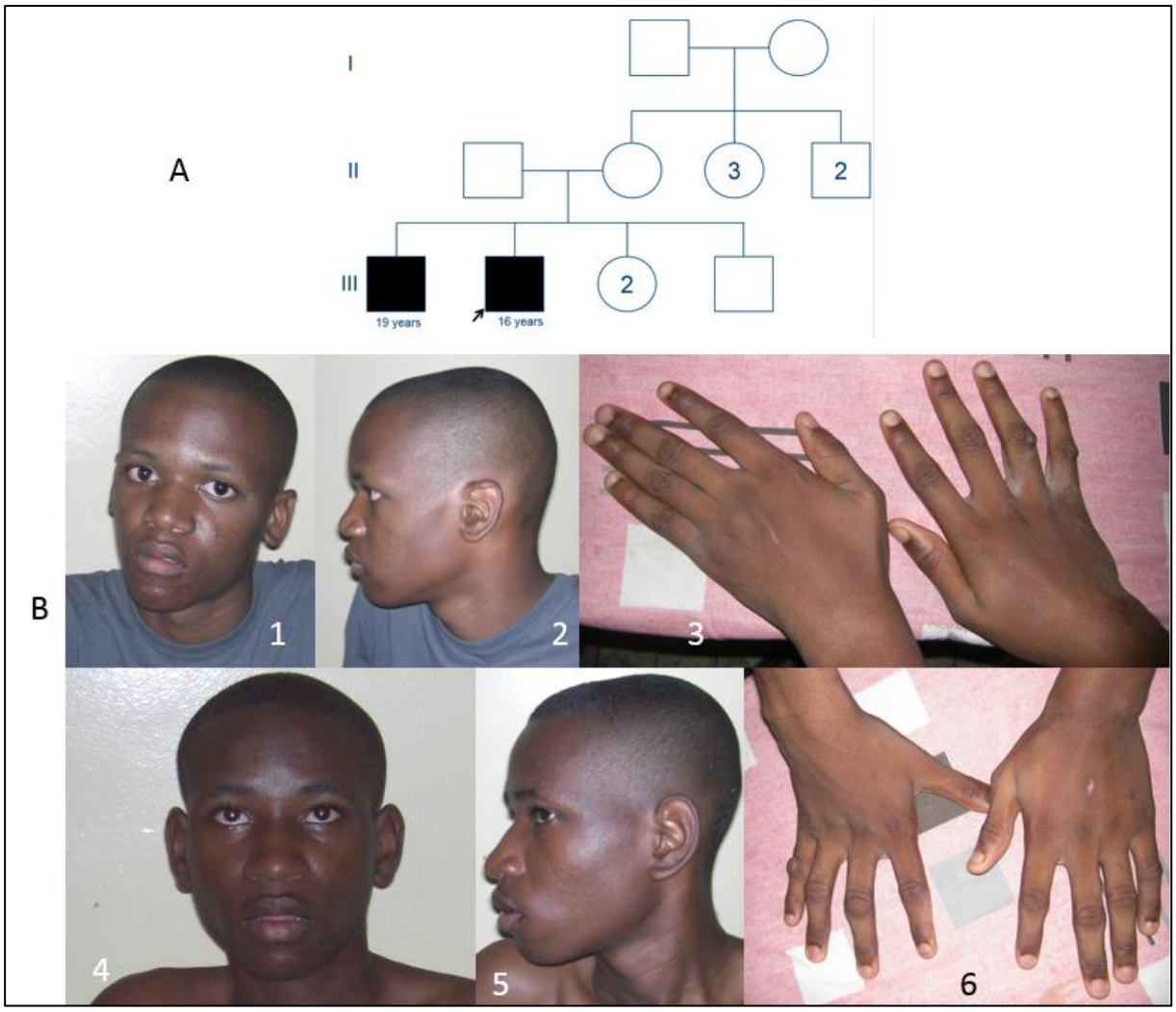

Figure 8. Pedigree and phenotype in Partington siblings. Long, triangular face in the index (B-1) and his brother (B-4), large and protruding ears in the index (B-1 and B-2) and in his brother (B-4 and B-5); dystonic position of hands less in the index (B-3) and, more pronounced in his older brother (B-6).

1.8.2.4 Noonan syndrome

This 6.15-year-old boy was born after a normal pregnancy as the second of four children from unrelated parents. He was the only male child. Family history was negative for ID. His father was diagnosed with a cardiac valvular problem at older age. The maternal grandfather had a heart disorder and a maternal uncle has a pacemaker. The patient was born with pulmonary valve stenosis, surgically corrected. He had speech problems, autistic like behaviour and mild ID with an IQ of 66. On clinical examination we noted a normal head circumference (52 cm, 0.2SD), relatively short stature (height 111 cm, -1.39SD) and normal weight (19.8 kg, 0.41SD). He was hypotonic, with drooling and a high arched palate (figure 9). He had ptosis, a high forehead, mild facial asymmetry, a high arched palate and special ears (over folded helices and upturned lobes). The toes were short and hands were slightly

swollen. Targeted resequencing of a panel of genes implicated in Noonan syndrome did not identify pathogenic mutation. This panel included the following genes: SOS1 (NM_005633.3) all the coding region; *RAF1* (NM_002880.3) exons 7, 14 and 17; *RIT1* (NM_006912.5) all the coding region; *KRAS* (NM_004985.3) all the coding region; *BRAF* (NM_004333.4) exons 4, 6, 11-16; *MEK1*/*MAP2K1* (NM_002755.3) exons 2 and 3; *MEK2*/*MAP2K2* (NM_030662.3) exons 2, 3 and 7; *HRAS* (NM_005343.2) exon 2. Fragile X testing, maternal X-chromosome inactivation profile and microarray-CGH were also normal.

Figure 9. Clinical features of the child with Noonan syndrome. Facial features (1-4) with ptosis, ears with upturned lobe and overfolded helices, high forehead. (5) Short toes, (6) oedematous aspects of the dorsal sides of the hands, (7) High arched palate.

1.8.2.5 Oculo-auriculo-vertebral spectrum.

This patient was a 7.85-year-old girl, the oldest of 3 children from healthy, unrelated parents. Pregnancy and delivery were uneventful. She functions at the level of moderate ID. On clinical examination, biometry was normal with OFC 53.5 cm (1.35 SD), weight 22 kg (-0.86 SD) and height 124 cm (-0.47 cm). We noted an asymmetry of the face with right-sided plagiocephaly, a prominent forehead, hypertelorism, epibulbar dermoid and coloboma of upper eyelid on the left eye, hypoplasia of the left malar bone and a small cheek on the left, bilateral pre-auricular tags, flat nasal tip, long philtrum, high arched palate, micro-retrognathia, short fingers and sandal gap (figure 10). There were no limitations in the mobility of her neck. Neurological examination was normal. All these features were consistent for the oculo-auriculo-vertebral spectrum (OAVS). Microarray-CGH was normal. ID is not a consistent feature of OAVS, but has been observed before (Beleza-Meireles, Clayton-Smith et al. 2014).

Figure 10. Facial features of the girl with oculo-auriculo-vertebral spectrum. Note the facial asymmetry, hypertelorism, hypoplastic malar bone and small left cheek (A), retrognathia, bilateral preauricular tags (B,C), epibulbar dermoid (D), coloboma of left eyelid (D), high arched palate (F).

1.8.2.6 Amniotic band syndrome.

This girl was 7.06-year-old and the only child of unrelated, healthy parents. Pregnancy and delivery were normal. At birth, limb reduction defects were observed for which a surgical intervention occurred at age 2. Details of this intervention are not available. Later, learning difficulties were noticed. Her OFC was 50 cm (-1.08 SD), weight 19 kg (-1.31 SD) and height 111 cm (-2.06 SD). We noted mild facial asymmetry with ptosis of the left eye and a high frontal hairline. On the hands, there were terminal reduction defects and partial syndactyly of the fingers. On the 5th finger on the left hand we noted a constriction ring. On the right foot, there was a severe reduction defect of the toes with syndactyly of the remaining parts of all toes. Neurological examination was normal (Figure 11). These features are consistent with amniotic band sequence (Moerman, Fryns et al. 1992). Intellectual disability has occasionally been observed in this condition. However, we were not able to perform brain imaging to exclude an underlying brain anomaly, as was described (Ruggieri, Spalice et al. 2007).

Figure 11. Clinical features of the patient with amniotic band syndrome. High frontal hairline (A and C), facial asymmetry with ptosis of the left eye (B), reduction defects of the toes and syndactyly (D), terminal reduction of the fingers, partial syndactyly and constriction crease (indicated with arrow) (E).

1.8.2.7 Acquired causes of ID.

In 8 patients, a tentative diagnosis of an acquired cause of their ID was made, based on a combination of findings, i.e. a clear history of an exceptionally severe central nervous system insult (meningitis, coma, severe birth injury), and at least two out of three of the following features: otherwise unexplained macrocephaly or microcephaly, epilepsy or severe neurological signs (spasticity, hypotonia or dystonia). In none of them, brain imaging could be performed.

1.8.3 Genome-wide screening with microarray-CGH

In 105 patients chromosomal microarray-CGH was performed, including 2 patients without a clinical diagnosis, the Noonan syndrome patient and the patient with OAVS. Also the patients with a suspected acquired cause were included, given the possibility of an underlying genetic cause (see chapter 4.3 for an illustration of this in the case with Williams syndrome). For economic reasons, we applied a protocol that permits the use of stripped slides. We re-used CytoSure™ ISCA v2 array 8x60k format (OGT, Oxford UK) slides, used once before.

1.8.3.1 Validation aCGH using CytoSure™ ISCA v2 array 8x60k stripped slides.

First, we compared the QCMetric in our experiment to those recorded when 3 slides were used for the first time and calculated the Paired Wilcoxon Ranked Sum Test P-Value. Recorded values for all QCmetrics were within normal ranges. There was no significant difference between the first and the second hybridization for DLRSpread ($p= 0.1232$), Signal Intensity (Red signal $p= 0.9102$ and Green signal $p = 0.9102$) and Non-Uniform Features ($p = 0.1755$) whereas the difference was significant for Background Noise (Red signal $p = 0.003906$ and Green signal $p = 0.05469$), Signal to Noise Ratio (Red signal $p = 0.003906$ and Green signal $p = 0.05469$) and Signal Reproducibility (Red signal $p = 0.003906$ and Green signal $p = 0.05469$). We concluded that the stripping process increased background but still within acceptable limits after one stripping cycle. Therefore we considered that our protocol for stripping the slides passed the quality criteria.

Next, we investigated the ability for stripped slides to detect known aberrations in tested patients. Our stripped slides did not miss any of the known aberrations greater than or equal to 400 kb (sensitivity 100 % at 400 kb). For CNVs with known clinical significance, the sensitivity was 100 % at 300 kb size. We concluded that stripped slides could reliably detect aberration greater than or equal to 400 kb.

1.8.3.2 Overview and discussion of microarray-CGH results in the patient cohort.

In 19 cases, the DNA samples did not pass the quality criteria. For these samples, the initial preparation of the mononuclear cells was done in Kinshasa, after which the cell pellets were transported to Leuven for further DNA preparation.

We considered CNV's to be causal when (a) they contain the critical region of known disease-related CNV's, or (b) when they comprise a dosage-sensitive gene known to cause ID when mutated (Breckpot, Thienpont et al. 2010). Thus, 10 CNV's were considered to be causal of the ID in 86 patients (11.6 %). All causal variants were confirmed using an independent technique, qPCR (in 9/10) or SNP-array (in 1 patient with a mosaic deletion).

Table 3. Causal CNVs identified in 10 patients

Num	Patient	ISCA nomenclature	Type	Size (bp)	Validation (tested gene)	inheritance
1	DRC-0033	arr 2p16.1(59,886,015-61,213,851)x1	Del	1,327,837	qPCR [*BCL11A*]	Mother normal, father NA
2	437575	arr [hg19] 2q24.3(165,828,304-166,616,001)x1	Del	787,698	qPCR [*SCN3A*]	Parents NA
3	DRC-0018	arr [hg19] 8p23.3-p23.1(61,749-11,985,357)x1	Del	11,923,609	qPCR [*GATA4*]	Mother normal, father NA
4	DRC-0136	arr [hg19] 13q14.2-q32.1(49,189,356-97,091,014)x1	Mos Del	47,901,659	SNP Array	Parents NA
5	DRC-0114	arr [hg19] 15q24.1-q24.2(72,963,962-75,535,357)x1	Del	2,571,396	qPCR [*PCSK2*]	Absent in both parents
6	437633	arr [hg19] 17p11.2(16,782,547-20,294,010)x1	Del	3,511,464	qPCR [*RAI1*]	Parents NA
7	437582	arr [hg19] 20q11.22-q11.23(33,186,305-34,775,792)x1	Del	1,589,488	qPCR [*GDF5*]	Parents NA
8	DRC-0040	arr [hg19] 20p13-q11.22(60,734-33,254,059)x3	Dup	33,193,326	qPCR [*PCSK2*]	Father normal, mother NA
9	416840	arr [hg19] 22q13.31q13.33(44,985,665-51,220,923)x1	Del	6,235,259	qPCR [*SHANK3*]	Parents NA
10	447761_4	arr [hg19] 15q11.2(22,698,520-23,217,513)x1	Del	518,994	qPCR [*CYFIP1*]	Paternal

NA: Not Available

1.8.3.3 Clinical and genetic description of the causal chromosomal imbalances

In the following sections, we describe patients with submicroscopic aberrations and provide a short discussion based on the literature.

Deletion chromosome 2p16.1

Patient DRC-0033 was a 14.89 years-old girl from unrelated, healthy parents. Pregnancy and delivery were uneventful. Her development was characterized by hypotonia, feeding difficulties and motor delay. She had seizures until the age of 3. Learning difficulties became apparent and she functions at a level of severe ID. On clinical examination the OFC was 51.5 cm (-1.73 SD), weight 45.9 kg (-0.73 SD) and height 154 cm (-1.2 SD). We noticed a narrow forehead, upslant of the eyes, ptosis, protruding lips and small mouth and chin, a remnant of post axial polydactyly (right hand), a left single palmar crease, pes planus and short toes (figure 12). *FMR1* testing showed 31/31 CGG repeats and XCI a 72.97 % pattern. She carried a 1,327,837 bp deletion on chromosome 2p16.1, arr [hg19] 2p16.1(59,886,015-61,213,851)x1. This deletion was not detected in the mother and the father was not available. This region contains the refseq genes *BCL11A*, *PAPOLG*, *REL* and 3' end of *RUS10*.

Several microdeletions in this region have been described associated with ID (Balci, Sawyer et al. 2015). The critical region for ID encompasses *BCL11A* and this was confirmed by the finding of a small deletion limited to *BCL11A* in a boy with ID (Peter et al., 2014). The deletion in the present case closely matches those reported by Hancarova et al. (2013) and case 1 of Piccione et al. (2012) (Piccione, Piro et al. 2012; Hancarova, Simandlova et al. 2013). They share severe ID and all three have a (relative) microcephaly and absence of major malformations. There is no obvious common facial dysmorphism. The child with only a deletion of the BCL11A gene had only mild ID and no microcephaly, suggesting that other genes in the deleted region contribute to these phenotypes.

Figure 12. Clinical features of the patient with cryptic 2p16.1 deletion. Note: narrow forehead, upslant of the palpebral fissures, ptosis (A), protruding lips and small mouth and chin (A-B), a remnant of post axial polydactyly on right hand (D), a left single palmar crease(D), short toes (E).

Deletion chromosome 2q24.3

Patient 437575 was a 10.40 years old boy, the eldest of two sons, and born to unrelated healthy parents. There was no record of pregnancy or birth complications, ID in the family or seizures in the patient. He had speech and learning disability. He was not assessed for IQ. He presents hand biting. On clinical examination, head circumference (54 cm, 0.36 SD), weight (41 kg, 1.01 SD) and height (143 cm, 0.38 SD) were normal. He had a truncal obesity, mildly hypoplastic thumbs, clinodactyly of the 5th toes and short toenails (figure 13). Conventional karyotyping was normal and *FMR1* assay detected 27 CGG repeats. Microarray-CGH detected a 787,698 bp deletion, arr [hg19] 2q24.3(165,828,304-166,616,001)x1, encompassing 3 RefSeq genes (*SCN3A, SCN2A, CSRNP3*) and few exons of *GALNT3*. The parents were not available for analysis. Other patients have been reported with ID who carried a deletion of *SCN2A* and *SCN3A* (Chen, Lin et al. 2010; Bartnik, Chun-Hui Tsai et al. 2011; Celle, Cuoco et al. 2013). This was variably associated with autism and seizures. Therefore, this deletion explains the phenotype of the patient.

Figure 13. Phenotype in patient with del2q24.3. Note truncal obesity (A), normal facial appearance (B) mildly hypoplastic thumbs (C), clinodactyly of the 5th toes and short toenails (D).

Deletion chromosome 8p23.3-p23.1

DRC-0018 was an 8.31 years-old girl and only child. Medical history of the father was not available. The mother had hearing problems. Pregnancy was uncomplicated but at birth she presented with neonatal distress and was treated in a neonatal intensive care unit. At age 2 months, a congenital heart defect, not otherwise described, was diagnosed, but follow-up was discontinued after age 4 years. She started crawling at 12 months and walking at 2 years after physical therapy. Later, she exhibited difficulties for grasping things such as a pen. Her speech was delayed and a hearing problem was notice by the family but she did not receive appropriate medical assessment. A psychiatrist followed her for behavioural problems. She functions at a level of mild ID. Her OFC was 50 cm (-1.42 SD) relatively small compared to her weight (29.5 kg, 0.55 SD) and height (131 cm, 0.27 SD). We observed mild strabismus, prominent nasolabial folds, flat philtrum, thin upper lip vermillion with absent cupid's bow, mild retrognathia and long fingers and toes (figure 14). Genetic assessment was normal for *FMR1* (31/32 repeats) but a highly skewed XCI profile was observed (8.97 %). Microarray-CHG revealed an 11.92 Mb deletion on the short arm of chromosome 8, arr [hg19] 8p23.3-p23.1(61.749-11.985.357)x1. The deletion was not present in the mother, the father was not available. The phenotype of deletions in the short arm of chromosome 8p, encompassing 8p23.1 has been extensively reported before (Claeys, Holvoet et al.

1997; Devriendt, Matthijs et al. 1999; Ballarati, Cereda et al. 2011). The phenotype in the present patient is consistent with this, including ID, behavioural difficulties, a relative microcephaly and a congenital heart defect, related to haploinsufficiency of the transcription factor *GATA4*.

Figure 14. Phenotype in patient with a del8p23.3-p23.1. Note mild strabismus (A), prominent nasolabial folds (A), flat philtrum (A&B), thin upper lip vermillion with absent cupid's bow (A&B), mild retrognathia (B) and long fingers and toes (C&D).

Mosaic deletion chromosome 13q14.2-q32.1

Patient DRC-0136 was 14.66 years-old when she was examined. She was born to healthy unrelated parents following an uncomplicated pregnancy and delivery. She had delayed development and her IQ was estimated at 63 (Bonhomme test). On clinical examination we noted a relative macrocephaly with OFC 56.6 cm (1.92 SD) for weight 40 kg (-1.58 SD) and a short stature with height 138 cm (-3.63 SD). There was a mild lower limb asymmetry. She had coarse facial features with a prominent forehead, depressed glabella, hypertelorism, strabismus, broad nose, flat philtrum, bilateral preauricular pits, short thumbs, deep palmar creases, dry palmar skin, bilateral clino-and brachydactyly of 5th fingers, swan neck deformity of fingers, swollen feet, a haemangioma on the anterior right upper leg and on the posterior left lower leg (figure 15). FMR1 analysis was normal and she had a moderately skewed XCI (16.85 %). Microarray-CGH revealed a 47.9 Mb mosaic deletion, arr [hg19] 13q14.2-q32.1(49,189,356-97,091,014)x1. This was confirmed using a SNP array with Illumina v2.1 which revealed the deletion in about 53 % of cells (figure 16). We did not find another case with the same

mosaic deletion in the literature. However, several patients are known with constitutive deletions in this region, but none of the same size as in the present patient. Nevertheless, the large size of the deletion and presence in over 50 % of white blood cells is a strong argument in favor of causality.

Figure 15. Phenotype in patient with mosaic del13q14.2-q32.1. Relative macrocephaly and asymmetric lower limbs (A), coarse face, strabismus, hypertelorism, broad nose (B), prominent forehead (C), preauricular pits (C-D) and peculiar shape of the ears (D), hypoplastic thumbs, brachydactyly of the 5th fingers and swan neck deformity of fingers (E-F), dry palm skin (F), swollen feet with short toes, limb hemangioma (H).

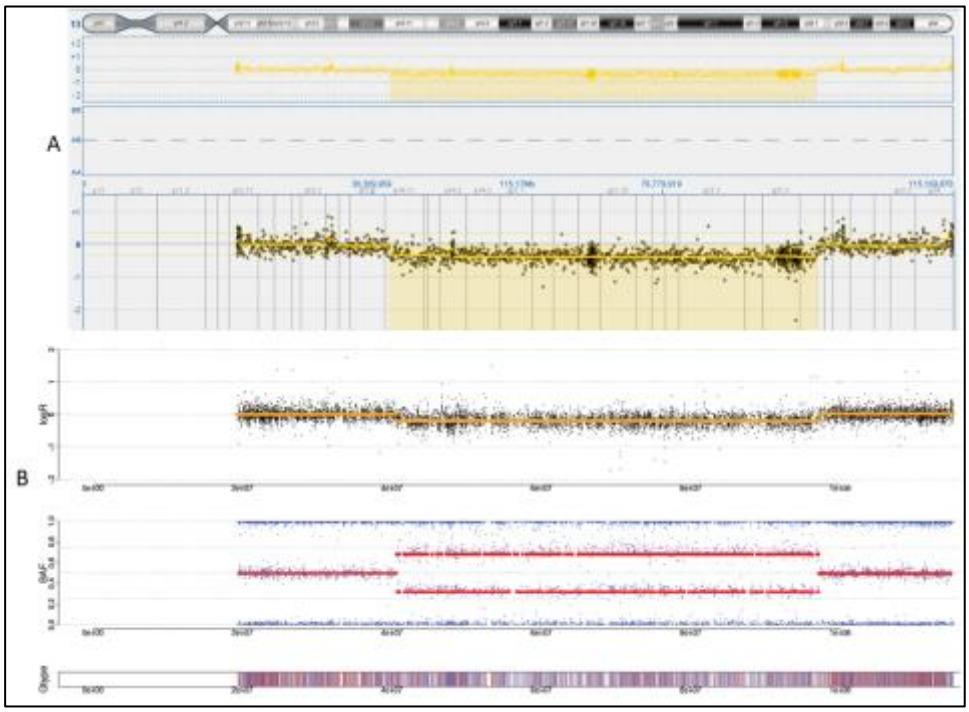

Figure 16. Views of the mosaic deletion with 8x60K ISCA Array (A) and Illumina v2.1 (B). The B-Allele Frequency is about 0.76.

Deletion chromosome 15q24.1-q24.2.

DRC-0114 was a 10.6 years boy, born to unrelated healthy parents. History of pregnancy and delivery were uneventful. He presented with speech regression from age 2 and psychological evaluation revealed moderate ID with autistic behaviour. On our clinical examination, OFC was 52.9 cm (-0.44 SD), weight 26 kg (-1.73 SD) and height 146 cm (0.65 SD). He had prominent nasolabial folds, a pointed small chin, small and protruding ears, multiple skin creases on the neck, long and slender fingers, brachydactyly of toes 4-5, a high sandal gap and phalangeal joint hyperlaxity (figure 17). FMR1 analysis was normal and maternal XCI profile was 44.99 %. Microarray-CGH identified a 2.57 Mb deletion arr [hg19] 15q24.1-q24.2(72,963,962-75,535,357)x1, de novo. This deletion corresponds to the recurrent 15q14 deletion syndrome, with breakpoints in LCR A and C (Mefford, Shur et al. 1993; Mefford, Rosenfeld et al. 2012). This is a known cause of ID, variably associated with major malformations and multiple minor anomalies. The reported facial features in other cases are not sufficiently distinct to allow a meaningful comparison with the present patient.

Figure 17. Phenotype in patient with Del15q24.1-q24.2. Prominent nasolabial folds, pointed chin, small and protruding ears (A), multiple skin creases on the neck (B), long and slender fingers (C), brachydactyly of toes 4-5, high sandal gap and phalangeal joint hyperlaxity (D).

Deletion of chromosome 17p11.2 (Smith-Magenis syndrome).

Patient 437633 was a 5.14 years old boy. He was the oldest of three sons born to unrelated parents. Pregnancy and birth were normal. He developed mood and behaviour problems characterized with self-injuries. His speech was limited, and he functions at a level of severe ID. On clinical examination, OFC was 50.2 cm (-0.78 SD), weight 17 kg (-0.74 SD) and height 104 cm (-1.19 SD). The face was triangular and hypotonic. There was a short bulbous nose with tented upper lip, upslant of the palpebral fissures, a short philtrum, and ears were dysplastic. He had scars from self-injuries on the front and on both hands (figure 18). Conventional karyotype and FMR1 analysis were normal. Microarray-CGH identified the recurrent 3.5 Mb Smith-Magenis deletion, arr [hg19]17p11.2(16,782,547-20,294,010)x1. The clinical phenotype of the present patient are in agreement with those observed in this well-known microdeletion syndrome.

Figure 18. Phenotype in patient with Smith-Magenis syndrome. (A): the facial hypotonia, short, bulbous nose, tented upper lip with short philtrum and upslant of the palpebral fissures; (B&C) short toes; (D&E) Scars on the hands due to self-injury (hand biting).

Deletion chromosome 20q11.22q11.23

Patient 437582, a 15.62 years-old boy was the oldest of 6 children from unrelated healthy parents. His development was characterized by pervasive behaviour and learning problems. He has an IQ of 51 (Bonhomme). Clinical examination revealed an OFC of 56.2 cm (0.49 SD), weight 46 kg (-1.56 SD) and height 152 cm (-2.48). He had hypertrichosis, a square chin, hypertelorism with downslant of the eyes, camptodactyly of the 5th fingers, brachydactyly of toes 4-5 and metatarsus adductus (figure 19). Karyotype and FMR1 testing were normal. Microarray-CGH showed a 1.58 Mb deletion on chromosome 20q11.22q11.23: arr [hg19] 20q11.22-q11.23(33,186,305-34,775,792)x1.

Deletions in this region have been reported before. The deletion encompasses *GDF5*, and haploinsufficiency is known to cause brachydactyly type C.

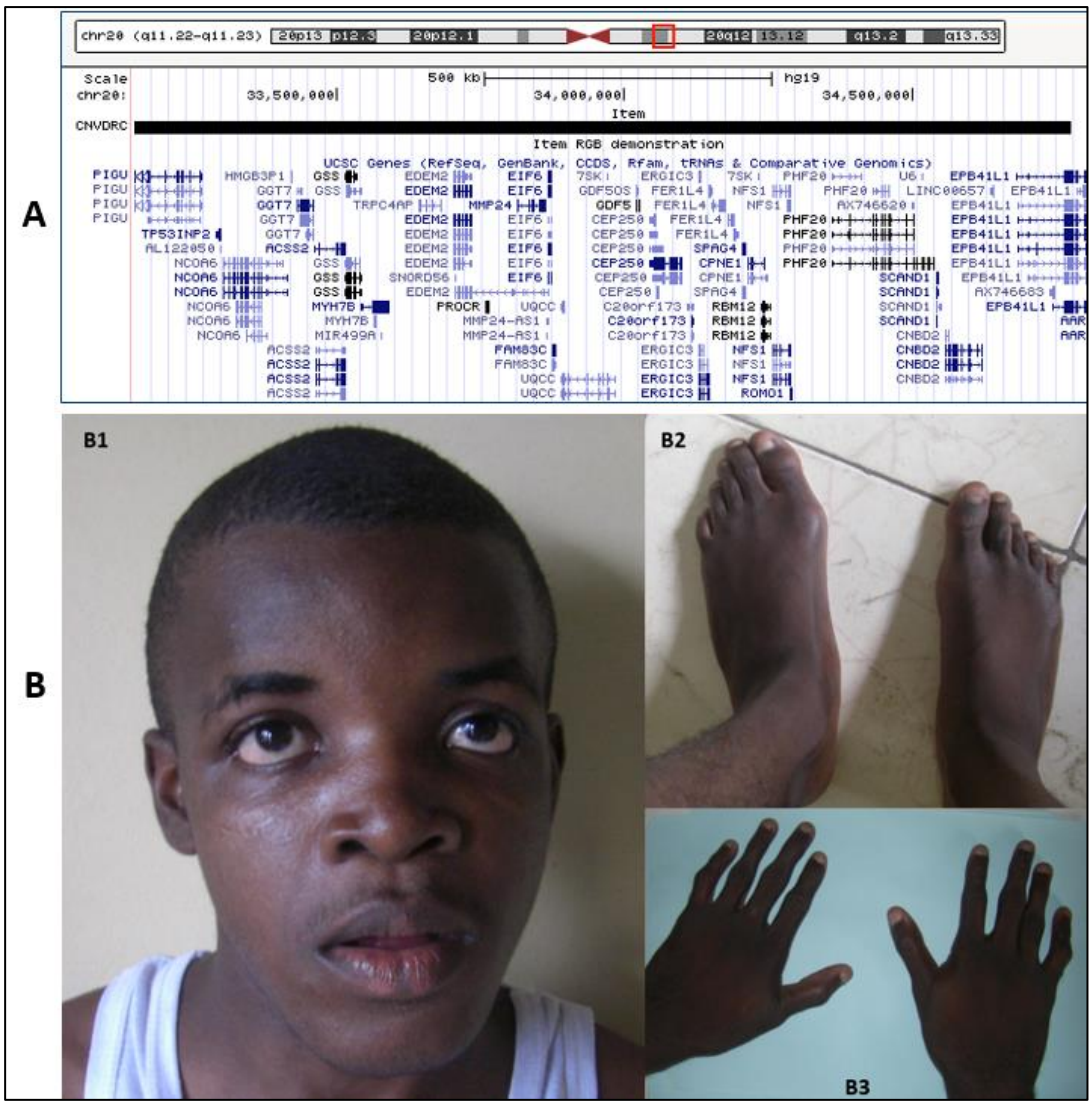

Figure 19. Panel A shows the deleted region and genes, including *GDF5*. Panel B displays the phenotype in the patient with del20q11.22q11.23. (B1) Hypertelorism, dowsnlant of eyes and square chin; (B2) brachydactyly of toes 4-5 and metatarsus adductus); (B3) camptodactyly of the 5th fingers.

Duplication 20p13-q11.22

DRC-0040, a 17.7 years-old boy is the youngest of 7 children, born to unrelated, normal parents. After a normal pregnancy, he presented with neonatal distress at birth. The development was delayed, and he presented speech regression at the age of 2 years. He has severe ID and autistic behaviour. On clinical examination, OFC was 57 cm (0.53 SD), weight 54 kg (-1.44 SD) and height 184 cm (1.13 SD). There was marked facial dysmorphism with a low set anterior and posterior hairline, thick and arched eyebrows, ptosis, a broad nose with broad nasal ridge, flat nasal tip, broad and everted nostril, short philtrum, thick and everted vermilion of the lips. There was camptodactyly and calluses on the 5th fingers and foetal fingertip pads (figure 20). FMR1 testing was normal. Microarray-CGH detected a 33.19 Mb interstitial duplication of chromosome 20p13q11.22: arr [hg19]20p13-q11.22(60,734-33,254,059)x3. Our patient presented features such as microcephaly, macrostomia, large ears similar to mosaic trisomy 20 patients 1 and 2 reported by Willis et al., 2008 (Willis, Bird et al. 2008).

Figure 20. Phenotype in patient with dup20p13-q11.22. (A) low anterior hairline, thick and arched eyebrows, ptosis, broad nose with broad nasal ridge, flat nasal tip and broad and everted nostrils, short philtrum, thick and everted lip vermilion, (B) low set posterior hairline, (C&E) camptodactyly and callus on 5th finger, (D) foetal pads.

Deletion 22q13.31q13.33

Patient 416840, aged 5.14 years, was the oldest boy of 3 children born to unrelated healthy parents. Family history was negative for ID. His development was delayed with regard to speech and motor milestones. He exhibit drooling, autistic behaviour and severe ID. On clinical examination, we noticed a relatively small head with OFC of 49 cm (-1.63 SD) with weight 20 kg (0.51) and height 118 cm (1.79 SD). He had a global hypotonia. He had dysmorphic features with arched eyebrows, long palpebral fissures, a short and bulbous nose, tented upper lip, and large ears. The fingers and toes were long and slender (figure 21). Karyotype and FMR1 analysis was normal. Microarray-CGH showed a 6.23 Mb deletion on terminal chromosome 22, arr [hg19] 22q13.31q13.33(44,985,665-51,220,923)x1. The terminal 22q deletion is a well-known entity. The presentation in our patient matches the phenotype of the syndrome which encompasses neonatal hypotonia, global developmental delay, normal to accelerated growth, absent to severely delayed speech and minor dysmorphic features a principal manifestations (Phelan, Rogers et al. 2001; Havens, Visootsak et al. 2004; Manning, Cassidy et al. 2004; Phelan 2008; Sarasua, Dwivedi et al. 2011; Phelan and McDermid 2012). *SHANK3*, one of the genes deleted in our patient, is thought to be the critical gene implicated in the neurodevelopmental phenotype (Wilson, Wong et al. 2003).

Figure 21. Phenotype in patient with a terminal 22q deletion. Note the global hypotonic habitus (A-B), long hands and feet (A), arched eyebrows, long palpebral fissures, short and bulbous nose, tented upper lip (B), large peculiar ears (C).

Deletion 15q11.2

Patient, 447761, was 17.65 years old, the third of 7 children born to unrelated healthy parents. Pregnancy and delivery were normal and there was no record of ID in the family. His development was reportedly normal until the age 13. Then parent noticed school problems, pervasive behaviour and increased mood instability. At the time of examination, he received treatment with multiple drugs including Haloperidol, Valproic acid, Clomipramine, Trihexyphenidyl and Promazine. On examination he was macrocephalic, with OFC 62 cm (3.8 SD) weight was 75.3 kg (0.72 SD) and height 182.5 cm (0.92 SD). Apart from thick eyebrows and a sandal gap, his appearance was normal (figure 22). FMR1 analysis was normal. Microarray-CGH revealed the presence of a 518.99 kb deletion on chromosome 15q11.2, arr [hg19] 15q11.2(22,698,520-23,217,513)x1. This corresponds to the recurrence BP1-BP2 deletion, including the gene *CYFIP1*. This microdeletion is a known risk factor for developmental disorders, including ID, autism spectrum disorders, schizophrenia and epilepsy (De Wolf, Brison et al. 2013). The deletion was also present in his father. This is in line with the observation that the vast majority of del15q11.2 are inherited, mostly from a normal parent. For this reason, and because of a high incidence in the normal population, this deletion is considered to be a susceptibility locus for ID, with an estimated penetrance of 10 %.

Figure 22. Phenotype in patient with the del15q11.2. Macrocephaly. Thick and straight eyebrows (A), normal hands (B) and sandal gap (C).

1.8.4 Fragile-X syndrome.

In the 105 patients tested with microarray-CGH, 25 girls and 80 boys, the size of the FMR1 CGG repeat was determined. Among the 130 alleles, not a single premutation or full mutation was detected (figure 23). Allele sizes ranged from 18 to 48 and the mean was 28.55 ± 2.84. Two patients presented with intermediate size alleles (47 and 48 repeats). Please refer to chapter 4 for a more detailed discussion of the FMR1 analysis.

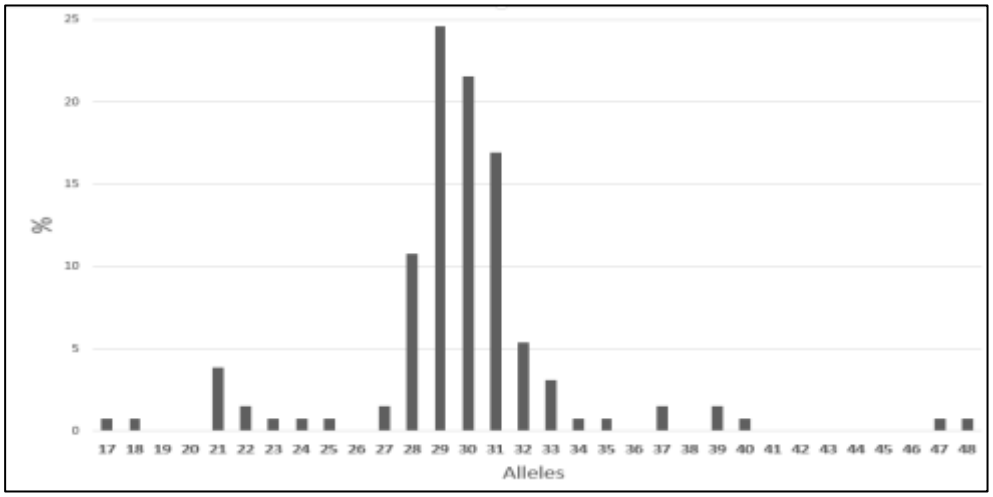

Figure 23. Distribution of CGG repeat alleles

1.8.5 X-chromosome inactivation analysis to identify X-linked ID.

From our cohort of individuals with ID we investigated X chromosome inactivation (XCI) profile in all 27 females indexes, as well as 41 mothers of male indexes from whom DNA was available. Of 41 mothers of boys with ID tested, 3 (7.32 %, CI: 1.89 - 18.63) had a highly skewed XCI (i.e. ≥90%) (figure 24). Three mothers (7.32 %) had moderate skewed XCI (i.e. 80%≤XCI<90%), 8 (19.51 %) had mild skewed XCI (70%≤XCI<80%) whereas 27 (65.85 %) showed random X-inactivation (i.e. <70). Among the 27 female indexes with ID, we detected 3 (11.11 %) with a highly skewed X-inactivation. Five (18.52 %) had moderate and mild skewing, respectively; and 14 (51.85 %) had random X-inactivation.

The proportion of mothers with highly skewed XCI is not significantly different from the normal population of adult females, where highly skewed X-inactivation was observed in 3.6 % (22 out of 415 females) (Amos-Landgraf, Cottle et al. 2006). This is probably due to the small size of our sample. However, when we consider both mothers and girls, 6 cases of highly skewed XCI were detected out of 68 individuals tested (8.82 %, CI: 3.65 - 17.45). Our prevalence is statistically higher compared to 1.8 % general proportion reported by Amos-Landgraf (Amos-Landgraf, Cottle et al. 2006). Both the small size of the population and the selective character of the sample may explain the apparent enrichment of skewed XCI in this cohort.

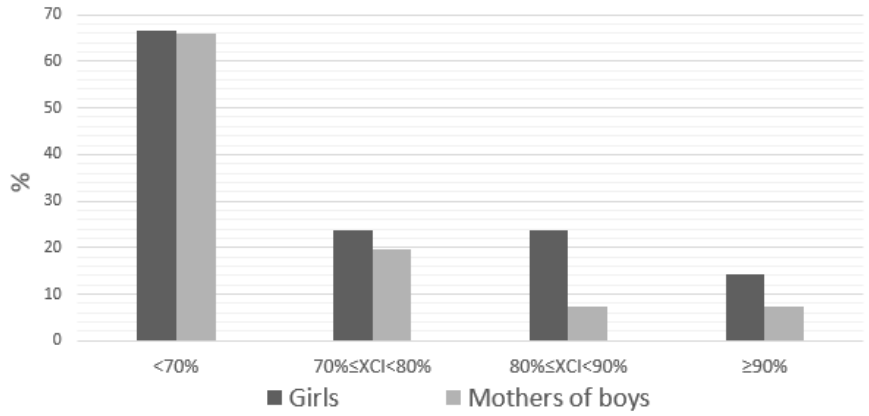

Figure 24. Distribution of X-inactivation patterns among girls with ID and mothers of boys with ID.

Case descriptions

We identified 3 boys with highly skewed X-inactivation in their mother (n = 3) and 3 females with ID and highly skewed X-inactivation. The clinical data are described below.

Case 1.

This boy was clinically examined at age 6.97 years. He is the only child of healthy, unrelated parents, and born after a normal pregnancy and delivery. Family history was negative with regard to ID. He presented with autistic behaviour and severe ID. On clinical examination his OFC was 51.7 cm (-0.06 SD), height 120 cm (-0.29 SD) and weight 19 kg (-1.46 SD). He was mildly dysmorphic, with underdeveloped ear lobes, long fingers and bilateral extra palmar skin creases on fingers 2-4. Neurological examination was normal. Microarray-CGH was normal. His mother had a highly skewed XCI pattern (91.76 %). Exome sequencing was performed.

Case 2.

This boy, aged 6.78 years, was the 5th child of healthy, unrelated parents. He has five healthy sisters. Family history was negative regarding ID. Pregnancy and delivery were normal. He presented delayed speech with first words pronounced at age 4 years. From the age of 2 years he had seizures, under control with medication. He was shy and friendly but had episodes of self-biting and uncontrolled appetite. He was reported to be difficult to assess for IQ. On clinical examination we noted microcephaly (head circumference 49 cm, -2.11 SD), normal height (110 cm, -1.89 SD) and weight (19.9 kg, -0.86 SD). He had a long face, high forehead, ptosis, downslant of the eyes, large ears, small and bulbous nose, everted lower lip, bilateral clinodactyly of the fifth fingers and toes, as well as scars resulting from hand biting. Microarray-CGH was normal. The mother had an XCI pattern of 92.75 %. Exome sequencing was performed.

Case 3.

This 6.51-year-old boy was born after an uneventful pregnancy and birth as the second of 3 children. His parents were healthy and unrelated, and family history was negative regarding ID. He had a normal motor development. Since the age of 3 years he presented seizures and under control with Sodium Valproate, Lamictal and Clonazepam. Language development was delayed, and when examined at the age of 6.51 years, he was unable to make sentences. He had learning difficulties with an estimated IQ of 57 (Borel-Maisonny test). He was hyperactive with attention deficit and presented gaze avoidance. He was diagnosed with autism based on the F84.0 [299.00] instrument from DSM IV. On clinical examination head circumference was 51 cm (-0.63 SD), height (114 cm, -0.83 SD) and weight (30.1 kg, +1.90 SD). His body mass index was 23.2 (+2.58 SD). He had a high frontal hairline, low set ears and a depressed nasal tip. His mother presented skewed X-inactivation (91 %). We did not have sufficient good quality DNA to proceed with exome sequencing.

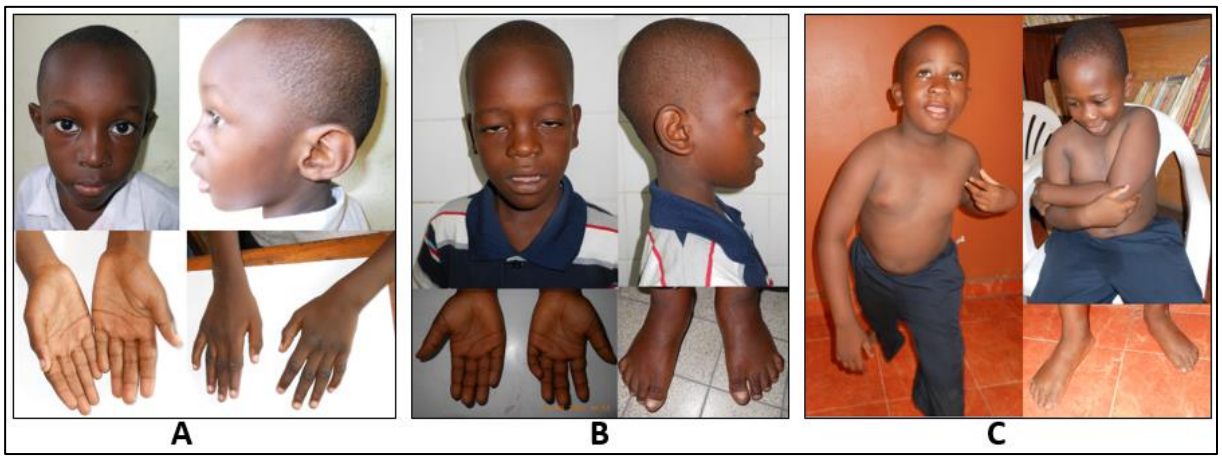

Figure 25. Clinical feature in patients DRC-0042 (case 1, Figure 25-A), DRC-0108 (case 2, Figure 25-B) and DRC- DRC-0118 5 (Case 3, Figure 25-C).

Case 4.

This girl with highly skewed XCI (5.18 %) was born from a mother with ID. Pregnancy and delivery were normal. She presented delayed speech development and seizures from the age of 2. She has a friendly behaviour. Intelligence was not assessed. On clinical examination at age 11.65, we noted severe microcephaly (OFC 48.5 cm -3.29 SD), weight 30 kg (-1.61 SD) and height 150 cm (0.18 SD). She had long fingers with extra skin creases on fingers 2-4, bilaterally. Microarray-CGH was normal. Her mother had moderate ID with speech problems but normal biometry, head circumference of 56cm (0.96 SD), height 159.5 cm (-0.59 SD) and weight 51 kg. She had long fingers, but clinical examination was otherwise unremarkable. X-inactivation patterns in her mother was not assessed. Exome sequencing was done in the index.

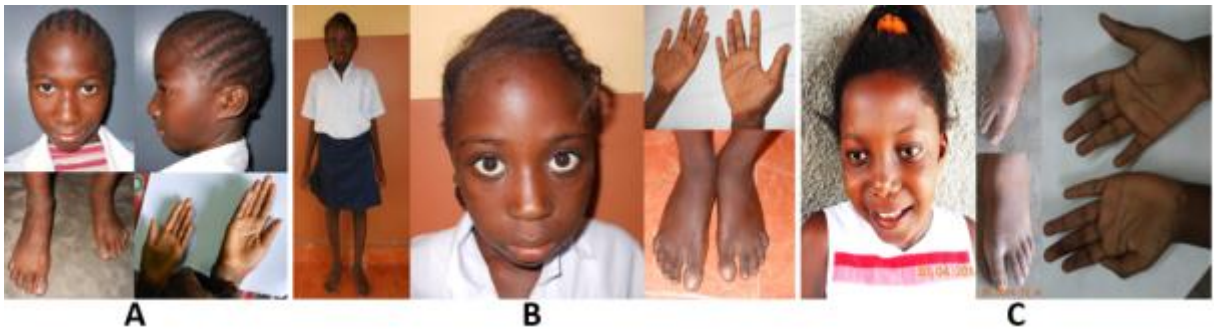

Figure 26. A=Clinical presentation in patients DRC-0032 (case 4), B=DRC-0154 (case 5) and C=DRC-0018 (case 6). Detailed dysmorphic description is made above.

Case 5.

This girl was born from a mother who also had ID. Pregnancy was uneventful and delivery was normal. She walked at age 12 months, first words came at age 4-5 years. When she started school, learning difficulties were apparent and her IQ was estimated at 53. She had attention deficit and limited social interactions. Clinical examination at age 11.68 revealed a global growth delay: microcephaly (OFC 50 cm, -2.21 SD), weight 22 kg (-3.76 SD) and height 129 cm (-3.39 SD). She had long fingers and toes with shortening of the 5th toes. She had a small chin. Microarray-CGH was normal. She has a skewed XCI (94.49 %). No sufficient good quality DNA was available for exome sequencing.

Case 6.

This girl carries a deletion 8p23.3-p23.1, and was already described above (DRC-0018).

1.8.6 Whole Exome Sequencing

We selected three cases (two boys and the girl without a causal CNV) from whom sufficient and good quality DNA was available, to perform whole exome sequencing (case 1, case 2 and case 4 described above).

In table 4, we show, for each of the three patients, the Sanger validated candidate variants that were retained after filtering, as outlined in the materials and methods section.

In Patient DRC-0042 (case 1) we did not identify any pathogenic X-linked variant. He was compound heterozygous for 2 variants in the AMINOACYLASE 1, ACY1 gene (MIM 104620). Each of his parent was carrier of one of the 2 variants. Mutations in *ACY1* are responsible for Aminoacylase 1 deficiency (609924) an autosomal recessive metabolic disease characterized by ID, seizures, hypotonia, motor delay and, in one family, sensorineural hearing loss (Van Coster, Gerlo et al. 2005; Ferri, Funghini et al. 2014). Interestingly, one of the 2 mutations (p.R386C) was previously reported and proven to down-regulate the activity of the Aminoacylase-1 (Sommer, Christensen et al. 2011). Confirmation of this

disorder can be done by metabolic testing on urine. However, this is not available locally for the moment.

Patient DRC-0108 (case 2) carried 2 variants in the SZT2 gene (MIM 615463), inherited from both parents. Mutations in this gene cause autosomal recessive epileptic encephalopathy, early infantile (MIM 615476), characterized by early-onset refractory epilepsy, with absent developmental milestones. Our patient has a history of early-onset seizures, which are now under control. The facial features are similar to those reported before, including downslant of the palpebral fissures, high forehead and ptosis (Basel-Vanagaite, Hershkovitz et al. 2013). Falcone et al., reported three brothers homozygous for a loss-of-function mutation in this gene, with a milder phenotype of non-syndromic mild to moderate ID, but without seizures, indicating large variability in expression of this condition. In our case, two missense variants were detected. The prediction tools do not support a pathogenic effect. Thus, it is not certain whether the found variants explain the phenotype in this patient.

In addition, we identified in case 2 a missense mutation in the X-linked gene TAF1:NM_004606:exon28:c.A4442G:p.N1481S, inherited from the mother. In a large screen of 405 families with XLID, 2 families were found to carry a mutation in this gene (Hu, Haas et al. 2015). However, no details about the phenotype were given. Because of its interactions with autism and ID genes such as *CHD8* and *MLL1*, it appears a good candidate for XLID. However, better genotype-phenotype correlations are needed as well as functional studies of the found mutations before one can draw definite conclusions. Reduced expression of this gene has been found in X-linked Dystonia-Parkinsonism (MIM 314250).

Table 4. Report of WES in the 3 patients from XCI study

Patients	Phenotype	Mode of inheritance	Gene	Type	NCBI RefSeq.	Exon	base change	AA change	Prediction*	Phenotypic concordance	Previously reported mutation	Segregation	Conclusion
Boy, Case 1 DRC-0042	autistic behaviour and severe ID, with underdeveloped ear lobes, long fingers and bilateral extra palmar skin creases on fingers 2-4	X-Linked	None										
		Autosomal Dominant	*COL4A2*	Missense	NM_001846	42	c.C3920G	p.P1307R	3	Possible	rs201627758	Maternally inherited	Excluded: inherited from normal mother
		Aut. Recessive Homozygous	*None*										
		Aut. Recessive Compound Heterozygous	*ACY1*	Missense	NM_000666	2	c.C49T	p.R17C	3	Yes	None	Not Maternal	Good candidate
			ACY1	Missense	NM_000666	15	c.C1156T	p.R386C	3	Yes	Reported as pathogenic: mutation PMID: 21414403	Maternally inherited	Good candidate
Case 2 Boy, DRC-0108	Severe ID, seizures, facial dysmorphism (high forehead & ptosis	X-Linked	*TAF1*	Missense	NM_004606	28	c.A4442G	p.N1481S	3	Yes – ID PMID: 25644381:	Not reported	Maternally inherited	Good candidate
		A. dominant	*GRIN2B*	Missense	NM_000834	13	c.G3389A	p.R1130Q	2	Possible Autism gene	rs148625092	Maternally inherited	Excluded: inherited from normal mother
		A. Recessive Homozygous	*None*										
		A. Recessive Compound Heterozygous	*SZT2*	Missense	NM_015284	27	c.T3880C	p.W1294R	0	Yes. infantile epilepsy, similar facial features Yes	rs72883814	Maternally inherited	Good candidate
			SZT2	Missense	NM_015284	53	c.G7402A	p.V2468I	0		rs139486476	Paternally inherited	Good candidate
Girl, Case 4 DRC-0032	Microcephaly, long fingers, bilateral extra skinfolds of fingers 2-4 bilaterally	X-Linked	*AMER1*	Missense	NM_152424	2	c.G1721A	p.R574Q	0	Yes	ClinVar: RCV000119998.1	inherited form affected mother	Excluded: No concordance
		Autosomal Dominant	*TUBB2B*	Missense	NM_178012	4	c.G553A	p.A185T	2	Yes	SNV present in a normal male and female control ClinVar: RCV000147838.1	absent in mother	Excluded: present in a normal controls

*Number of tools predicting the mutation as pathogenic or disease causing among the following: Mutation Taster, Polyphen2 and SIFT.

Patient DRC-0032 (Case 4), has highly skewed XCI and had a mother with ID. We filtered the exome data for X-linked and autosomal dominant inheritance. We identified a missense mutation in the AMER1 gene, inherited from her mother. This gene causes osteopathia striata with cranial sclerosis (MIM 300373) characterized by macrocephaly, cleft palate, mild learning disabilities, sclerosis of the long bones and skull. This phenotype did not match with our patient nor her mother. She carried a variant in the TUBB2B gene, which was previously reported in Clinvar as RCV000147838.1. This variant was not inherited from the mother, and we also found this in a normal control. Therefore, we believe this is not a pathogenic mutation.

In addition to the 3 patients from XCI assessment, two affected sisters also underwent WES. We hypothesized the presence of an autosomal recessive inheritance. They were born to unrelated parents. Pregnancies and births were uneventful. There was no family history of ID or psychiatric problems.

The index was 20.1 years. She had a delayed development: she sat alone at age 12 months and walked after 2 years of age. Speech consisted of unintelligible sounds only. She had fine motor difficulties, and was not able to dress or undress herself. At the age of 2 years she had episodes of seizures, under control with antiepileptic medication. She had aggressive behaviour with multiple lesions from self-injury. She had a normal biometry (OFC: 55 cm, 0.26 SD; height 165 cm, weight: 60 kg). We note a diplegic gait, coarse facial features, hypertelorism, ptosis, strabismus, a pointed chin, rigidity of the limbs and scars on the hands from hand biting.

Figure 27. Phenotype in sisters with ID and psychiatric behaviour. Index in A, B and C; her sister in D, E and F. Coarse face (A, B, D&E), hypertelorism (B&E), ptosis (B&E), strabismus (B&E), pointed chin (B&E), diplegic upper limbs (A&D), scars from self injury (B, C, E & F).

Her sister was 25.11 years. She had a history of neonatal distress. Likewise, she had a delayed motor development, impaired fine motor skills and unintelligible speech. Behaviour is as impaired as in the index. She did not present seizures. She is under psychiatric care for behavioural problems. Her biometry was normal (OFC: 57 cm, 1.64 SD; height: 160 cm and weight: 83.4 kg). Similar to her sister, she had a diplegic gait, coarse face, ptosis, strabismus, an everted lower lip, pointed chin, rigidity of the limbs and scars resulting from hand biting.

FMR1 testing and microarray-CGH were normal. XCI was not informative. Both of them underwent WES. We did not identify a causal mutation after filtering for autosomal recessive or dominant inheritance. We identified 2 compound heterozygous mutations in the *LINS* gene (implicated in autosomal recessive Mental Retardation 27): NM_001040616:exon6:c.G1373A:p.G458D and NM_001040616:exon5:c.A1000G:p.M334V. However, Sanger validation shows that both were inherited from the normal mother, thus excluding a causal role for this gene.

1.9 Discussion

We report the results of an etiological diagnostic study in 127 individuals with ID, recruited in Kinshasa, capital of DR Congo in Central Africa. Figure 28 (flow chart) gives an overview of the different steps in the study and results, according to different diagnostic categories. The results for individual patients were already discussed in the results section, and here, we discuss the general aspects of our results.

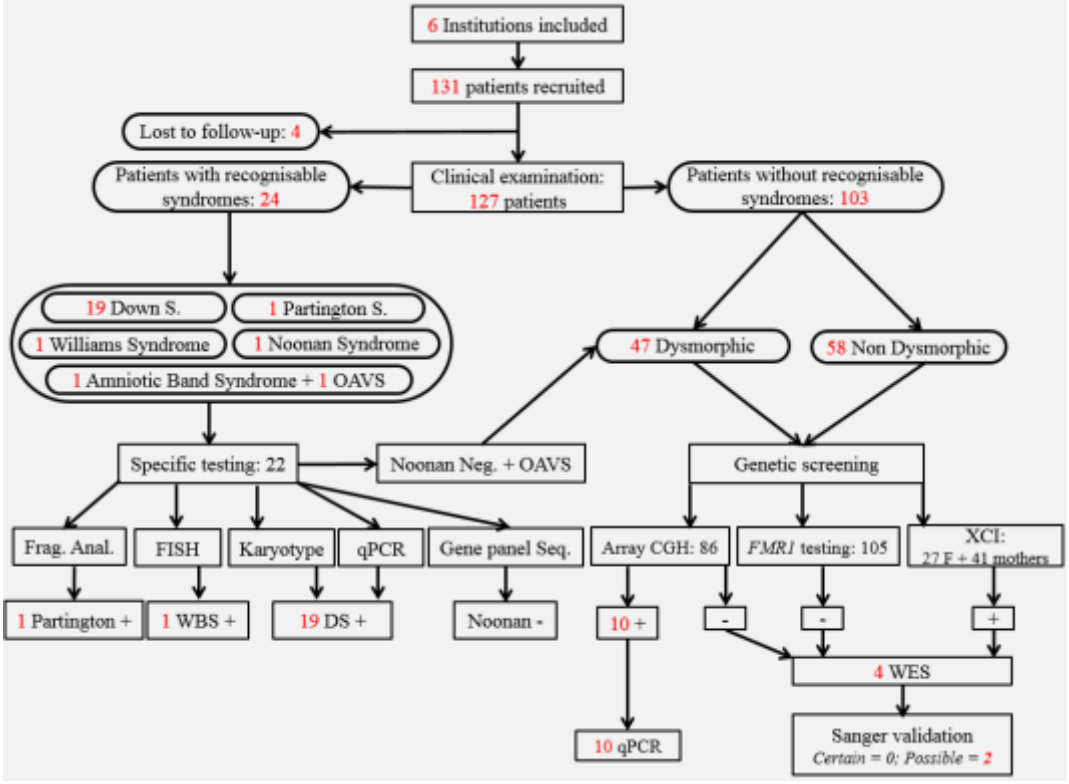

Figure 28. Overview of screening results. Detailed steps taken and number of cases at each step.

We used different strategies to identify the aetiology for the ID in this study. Clinically, we established a diagnosis in 24 out of the 127 patients (19 %). There were two disorders without a known genetic cause, amniotic band syndrome and oculo-auriculo-vertebral syndrome. Of the 22 cases clinically diagnosed, 21 (95 %) were confirmed by targeted genetic testing. In the child with clinically convincing Noonan syndrome, no mutation was detected in the panel of genes currently associated with this condition. To date, in approximately 15 % of Noonan syndrome cases, no genetic cause is identified (http://www.ncbi.nlm.nih.gov/books/NBK1124/).

In 8 patients, we suspected a probable acquired cause based on the birth history of trauma or severe neonatal distress, a history of brain cerebral infection at a young age, neurological sequel such as cerebral palsy, seizures and microcephaly. One of the limitations of our study is that in this setting, brain imaging is not routinely available. Therefore, brain imaging to support a suspected acquired

cause by demonstrating brain lesions is missing. Since an acquired cause may coexist with a genetic defect, the 8 patients were further studied by genetic screening tests, with normal results.

The high yield of clinical diagnoses demonstrates that a clinical genetic approach is still the basis of genetic diagnosis (Rauch, Hoyer et al. 2006).

According to the current guidelines genetic screening tests in unexplained ID include FMR1 testing and microarray-CGH (Moeschler and Shevell 2014). We did not identify a single case with Fragile X syndrome. This is discussed in more detail in chapter 4. By means of microarray-CGH, a pathogenic CNV was identified in 10/86 patients with good quality data (11.6 %). Of these, 9 were true causal CNV's, i.e. sufficient to cause the ID. In one individual, a del15q1.2 was identified, which is a low risk factor for developmental delay and ID.

Next, we performed a preselection of cases for further study by means of exome sequencing. In two siblings with syndromic ID, no causal variant was identified. Of the 68 female patients and mothers of affected boys, tested for XCI, 6 (13.6 %) presented with highly skewed X-inactivation. This proportion is close to 9.8 % previously reported (Amos-Landgraf, Cottle et al. 2006). We selected three cases for exome sequencing. In two of them, a possible genetic cause was identified. One boy was compound heterozygous for two variants in the *ACY1*, causing Aminoacylase 1 deficiency. Confirmation of this diagnosis is pending on metabolic studies. In the other boy, a likely causal mutation was identified in the TAF1-gene, a recently identified candidate gene for XL-ID. However, more clinical evidence and functional studies of the found variant are needed in order to confirm that this is a causal mutation.

Taken together, a genetic diagnosis was reached in 34/127 patients (26.8 %). When we include the 8 probably acquired causes and the results of the exome analysis, a diagnosis was obtained in 44 (34.7 %).

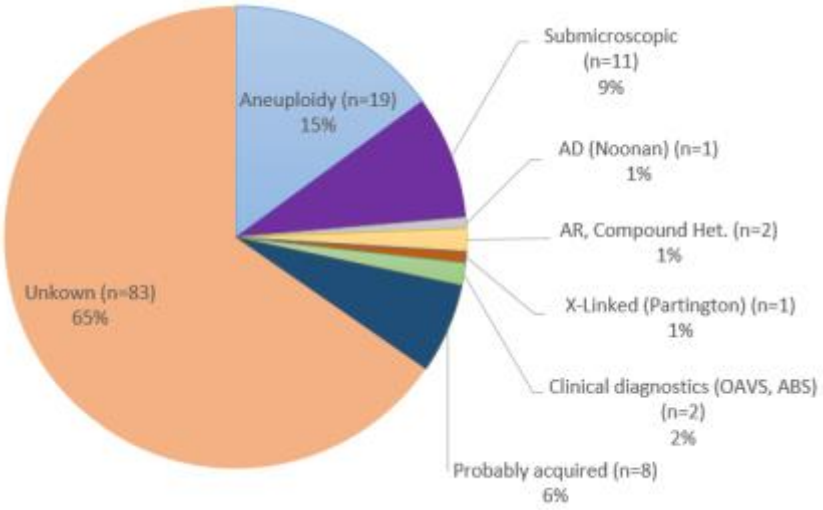

Figure 29. Distribution of different categories of etiological diagnoses

Altogether, genetic causes were identified in 34/127 patients, the majority of them chromosomal aberrations, including submicroscopic aberration in 11 patients. In addition, monogenic causes were identified in 3 patients (1 Partington syndrome patient and probably also in 2 patients by means of WES).

With 19 cases out of 127 (14.8 %; CI: 9.531 % - 21.97 %), Down syndrome is the most prevalent genetic cause in our cohort. In most series, Down syndrome is the most prevalent cause of ID (Devriendt, Holvoet et al. 2003; Rauch, Hoyer et al. 2006). However, our finding is higher than 9.2 % reported by Rauch et al. in Switzerland, and 7.1 % reported by Devriendt et al in Flanders. This could be due to the absence of prenatal screening program in DR Congo. Among the 19 Down syndrome patients, 1 of them presented with a less pronounced facial characteristics, and genetic testing was consistent with a mosaicism. Advanced maternal age is a well-known risk factor for the classical type of free trisomy 21 (Snijders, Sebire et al. 1995; Snijders, Sebire et al. 1995; Csermely, Czeizel et al. 2015). Also in our cohort, an advanced maternal age equal or above 35 years was observed in the majority of mothers of children with Down syndrome.

For the purpose of this study, we implemented a rapid and easy-to-handle qPCR which correlated with the karyotype and microarray-CGH results. We therefore suggest that qPCR assay is the method of choice for the initial genetic confirmation of a clinically diagnosis of Down syndrome in the DR Congo.

Eleven submicroscopic chromosomal imbalances were identified. Of interest, four of these are known, recurrent CNV's, mediated by flanking low copy repeats: Williams syndrome, Smith-Magenis syndrome, the del15q11.2 and the del 15q24. The phenotypes, even though only recognized clinically in the Williams syndrome patient, correspond to the descriptions in Caucasian patients, taking in to account the large variability often observed for these syndromes.

To identify monogenic causal defects, we applied different strategies including targeted testing for diagnoses suspected by clinical examination (Partington syndrome), screening for the fragile syndrome and an XCI assay to identify potential carriers of X-linked disorders, followed by whole exome sequencing.

The index with Partington syndrome as well as his older brother had the typical clinical presentation as described in Caucasian patients in the literature (Partington, Mulley et al. 1988; Gedeon, Partington et al. 1994; Cossee, Faivre et al. 2011). We could not find records of similar cases from Central Africa.

Although we did not detect a patient with fragile pre- or full mutation, we identified 2 carriers for the intermediate size allele. Since these represent susceptibility alleles, this suggests that the Fragile X syndrome does exist in Congolese patients, but that larger studies are needed to identify them.

In two sisters with a same phenotype of severe ID, behavioural problems and similar facial and neurological phenotype, we failed to identify a genetic cause by means a of exome sequencing.

The data we present concern children attending special schools or institutions in Kinshasa. For this reason, this is a biased study. In the local cultural and economic context, children with more severe ID and those with major malformations rarely attend school. In total, only 9 patients had a major malformation. In a similar study in Flanders, the likelihood of having a major malformation when there are 5 or more minor anomalies was 44 % (Devriendt, Holvoet et al. 2003). We compared the distribution of minor anomalies in our cohort to that of the Flemish study. Overall, the distribution was similar, except for a clear underrepresentation of the ones with multiple minor anomalies (Figure 30). In addition to economic and cultural selection, another reason for the smaller number of cases with major malformation in our series could be the limited chance to survive from life-threatening malformations in limited resource setting sub-Saharan Africa.

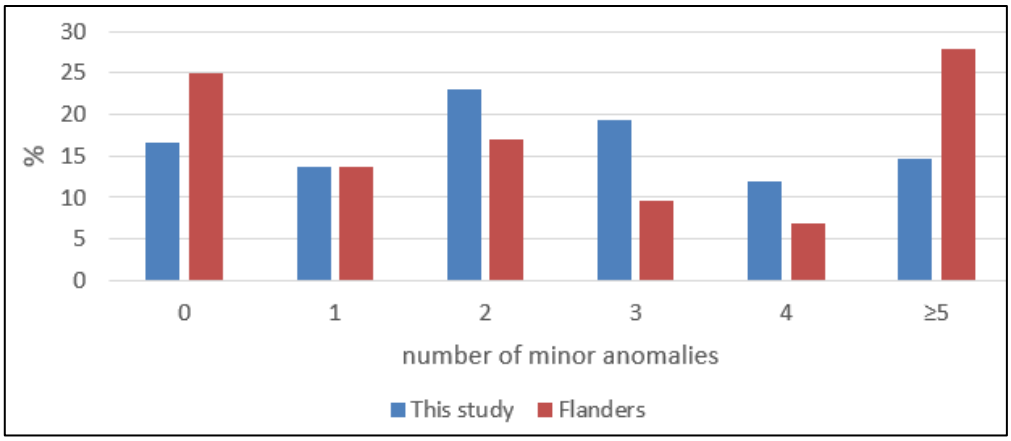

Figure 30. Comparison of proportion of minor anomalies in this study versus Flanders.

This is one of the first studies on genetic aspects of ID in a central African country. The results clearly show that the same range of genetic and environmental causes are identified compared to other societies. This is an important message for families of children with ID and caregivers, to refute commonly held ideas and traditional mystical believes on the causes of ID.

1.10 References

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Sequencing primer with M13 Tag (Red sequences)

AUTS2_Ex19_F: tgtaaaacgacggccagtTTGAGGGATCCTTACCGAGA

AUTS2_Ex19_R : caggaaacagctatgaccGATAGTGCATGCTGGGGAGT

COL4A2_Ex42_F : tgtaaaacgacggccagtCCAGAGACTGTCGCCTGA

COL4A2_Ex42_R: caggaaacagctatgaccGGGAGACCAAACACACCAG

ACY1_Ex2_F: tgtaaaacgacggccagtGTGCAGGAGCTCACCATTTC

ACY1_Ex2_R : caggaaacagctatgaccACCTTGGTCAGCAGCAGAGT

ACY1_Ex15_F: tgtaaaacgacggccagtCCCATTCATCAGGCTCTTTC

ACY1_Ex15_R : caggaaacagctatgaccAGGGGCAAAGGTTTAGGAGT

RELN_Ex_22_R: tgtaaaacgacggccagtTTCACTTACAGTGGGTGAGTAGACA

RELN_Ex22_F: caggaaacagctatgaccCATGCTATTGTGCTTAACTTGC

SHANK2_Ex2_F: tgtaaaacgacggccagtAGACCATTGGGAGCTACGTG

SHANK2_Ex2_R : caggaaacagctatgaccGCAGGGACAGTGAGCAAAAC

TUBB2B_Ex4_F: tgtaaaacgacggccagtGTCATACAGGGCCTCGTTGT

TUBB2B_Ex4_R : caggaaacagctatgaccCTTCCAGCTGACCCACTCTC

AMER1_Ex2_F : tgtaaaacgacggccagtGCCTTGAGAACTCTCCACCT

AMER1_Ex2_R: caggaaacagctatgaccCTGGGTCTCTCGGACTTGAG

TAF1_Ex28_F: tgtaaaacgacggccagtTCCAGTCAATGCAAAGGTTG

TAF1_Ex28_R : caggaaacagctatgaccGTCCAATCTAGTCTGAATTTAGGG

GRIN2B_Ex13_F : tgtaaaacgacggccagtGGCGTAAGCAGCAATATAAGG

GRIN2B_Ex13_R : caggaaacagctatgaccGTCGGTCAGGTCTACGTGCT

SZT2_Ex27_F : tgtaaaacgacggccagtTTTGCTGACAGCGGTAGATG

SZT2_Ex27_R : caggaaacagctatgaccCCCCCTTATCCATCATTCAA

SZT2_Ex53_F : tgtaaaacgacggccagtCTGGAGCCCAGAGACAAAAG

SZT2_Ex53_R : caggaaacagctatgaccTCTGGACACTGAGGCACAAC

LINS_Ex_R : tgtaaaacgacggccagtTGACCTTCTTAAAGCCTCATCT

LINS_Ex6_F : caggaaacagctatgaccTTTCCCCCAAATGATGAAAG

LINS_Ex5_F: tgtaaaacgacggccagtGCTTGCTGCTCTAAGGATCAC

LINS_Ex5_R : caggaaacagctatgaccTTCTCATCGCCTCCAGAATC

qPCR primers :

qPCR_CYFIP1_F_: GCTAGACACGGACAGACAGGA

qPCR_CYFIP1_R : GGGTGTGTGTGTCGAGTGA

qPCR_MEF2C_F : CCAGGCTTAAGTCCCACTGA

qPCR_MEF2C_R : GTGACAGCATTCCAGAAGCA

qPCR_NCAM2_Ex16_v2_F : TCGAAGAAGGAAAAGCTGCA

qPCR_NCAM2_Ex16_v2_R : CCCTCAGGTAAAGCACTCGA

qPCR_SCN2A_F: TGTAGGCCTGAAGACCATTGT

qPCR_SCN2A_R:TTGCCCATGAACAACTGCAA

qPCR_SHANK3_R: TCAGGTTGATATCGCTGGCT

qPCR_SHANK3_F : CCCCTTACCCCAGTACCATT

qPCR_GATA4_Ex7_F: CCTCCTCTGCCTGGTAATGA

qPCR_GATA4_Ex7_R: AAAATCCAACACCCGCTTCC

qPCR_MIP_Ex3_F : AGTTGTGGATGGGGACTCAC

qPCR_MIP_Ex3_R: GTGAGATGCGGTTGTCAAGG

qPCR_RAI1_Ex3_v2_F : AACATCTCCAACACCGTCCA

qPCR_RAI1_Ex3_v2_R : TGGACACGAGGTTCTTGACA

qPCR_PCSK2_Ex12_v2_F : AAGTCCATTTTGCTGAGCCG

qPCR_PCSK2_ex12_v2_R : TGAGTGGTCATGAAAGGCCA

qPRC_BCL11A_Ex2_F : CCTTCCCCTTCACCAATCGA

qPRC_BCL11A_Ex2_R : AACAATCGTCATCCTCTGGC

qPCR_GDF5_v1_F : CCAAGAAACGGGACCTGTTC

qPCR_GDF5_v1_R : GTCTTATCGTCCTGGCCAGA

SCREENING FOR THE FRAGILE X SYNDROME AMONG PATIENTS WITH INTELLECTUAL DISABILITY IN KINSHASA, DR CONGO

(Manuscript in preparation)

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1.11 Introduction

The Fragile X Syndrome (**FXS**) (OMIM 300624) is one of the most common monogenic causes of Intellectual Disability (ID). Its prevalence varies between 1/6,000 and 1/4,000 males in the general population (Turner, Webb et al. 1996; de Vries, Mohkamsing et al. 1999). Because of this high incidence, screening for Fragile X syndrome is considered essential in any child with developmental delay or intellectual disability (ID) (Moeschler and Shevell 2014). A variable prevalence is observed in different geographical parts of the world, but prevalence also depends on the clinical characteristics of the tested group, gender and sampling strategy (Crawford, Acuna et al. 2001). Only few studies have investigated FXS in Africa. In children attending schools for special education, Goldman reported a prevalence of 6.1 % of FXS among black Africans in South Africa (Goldman, Jenkins et al. 1998). Later, Crawford et al., reported a prevalence of 1/2,545 among African Americans in special education in the public school system of metropolitan Atlanta, USA (Crawford, Meadows et al. 2002). To date, there are no reports on FXS in schools for special education or clinics for children in Central Africa.

In at least 99 % of FXS cases, the genetic mutation is an expansion above normal ranges of the CGG repeats in the 5'-UTR in the promoter region of the *FMR1* (Fragile X Mental Retardation 1; OMIM: 309550). The remaining cases are explained either by chromosomal imbalances (e.g. deletions) or rare intragenic mutations (Tarleton, Richie et al. 1993; Albright, Lachiewicz et al. 1994; D'Hulst and Kooy 2009; Okray, de Esch et al. 2015). **Normal** alleles range from ~5 to ~44 repeats, **intermediate** alleles from ~45 to ~54 repeats, **premutation** alleles from ~55 to ~200 repeats **and full mutations** over 200-230 repeats (Maddalena, Richards et al. 2001; Monaghan, Lyon et al. 2013). Comparatives studies between African Americans and Caucasian Americans reported a significant difference in the distribution of common alleles with shorter alleles (20-23 repeats) as well as larger alleles (41-60 repeats) being less frequent in African Americans (Eichler, Hammond et al. 1995; Crawford, Zhang et al. 2000). Although the 29 and 30 CGG repeats are the most prevalent alleles in Africans, African Americans and Caucasians, the 29 repeats allele has a significantly higher frequency in black Africans whereas the 30 repeats allele is significantly more frequent in white Africans (Chiurazzi, Destro-Bisol et al. 1996; Crawford, Meadows et al. 2002; Essop and Krause 2013).

The FMR1 repeat is not a perfect tandem repeat, but interrupted by AGG trinucleotides at regular intervals. These appear to stabilize the repeat within normal ranges. Inversely, the instability increases with longer perfect repeats (Eichler, Macpherson et al. 1996). In Caucasians, the FMR1 expansion mutation is in linkage disequilibrium with specific alleles of flanking microsatellites (Chiurazzi, Destro-Bisol et al. 1996; Larsen, Vuust et al. 2001). African and African Americans exhibit greater variability of

polymorphisms around the FRAXA locus, in line with evolutionary origin of Homo Sapiens which initiated in Central Africa (Chiurazzi, Destro-Bisol et al. 1996; Crawford, Zhang et al. 2000; Peprah, Allen et al. 2010).

The FXS is associated with various clinical presentations depending on the number of repeats, sex and age (Oostra and Willemsen 2009). Hence, concepts such as "Fragile X Spectrum Disorder" (FXSD) or FMR1 related disorders have been proposed in order to include all *FMR1*-related phenotypes (Lozano, Rosero et al. 2014). The full mutation in young children manifests often with Attention Deficit Hyperactivity Disorder (ADHD) and/or Autism spectrum disorders (ASD) (Hagerman 2006; Sullivan, Hatton et al. 2006; Gabis and Kesner 2007). In older children, adolescents and adults the classic presentation consists of intellectual disability with an IQ < 50, associated to more characteristic behavioural and dysmorphic features (Hagerman, Amiri et al. 1991; Tuncbilek, Alikasifoglu et al. 1999; Lachiewicz, Dawson et al. 2000; Mirkin 2007; D'Hulst and Kooy 2009; Lozano, Rosero et al. 2014). Although none of the listed features is pathognomonic for FXS, their association has been used to efficiently screen for the FXS using clinical checklists (Butler, Mangrum et al. 1991; Laing, Partington et al. 1991; Lachiewicz, Dawson et al. 2000). These checklists are not intended to substitute molecular testing. Instead, they allow a fast and efficient selection of patients with a higher probability for having FXS (Hagerman, Amiri et al. 1991; de Vries, Mohkamsing et al. 1999; Behery 2008).

So far, no checklist has specifically been designed or adapted for the black African population. Indeed, craniofacial features in a population of African origin may show critical differences compared to those in Caucasian for certain genetic conditions, as was reported for the 22q11.2 deletion syndrome (McDonald-McGinn, Minugh-Purvis et al. 2005) and for FXS (Schwartz, Phelan et al. 1988). Such clinical variability points to the necessity to design population specific checklists or adapt existing tools to the population of interest. Examples of population specific checklists for FXS have been proposed for the Indian and Turkish population (Tuncbilek, Alikasifoglu et al. 1999; Guruju, Lavanya et al. 2009).

The present study aimed to gain insight into the prevalence of FXS and the distribution of CGG alleles, and to evaluate the usefulness of three selected checklists as a screening tool for FXS in patients with ID followed in specialised clinics and schools in Kinshasa, DR Congo.

1.12 Material and Methods

1.12.1 Patients

We recruited 127 index patients from 2 hospitals and 4 schools specialised for ID across Kinshasa in the Democratic Republic of Congo (DRC). Informed consent was obtained from parents or legal representatives. We conducted a standard clinical genetic examination which allowed the identification of 22 patients with a clinically recognizable syndrome including Down syndrome (n = 19), and respectively one patient for Williams syndrome, Partington syndrome, amniotic band syndrome and Oculo-Auriculo-Vertebral Spectrum. One patient had a clinical diagnosis of Noonan syndrome, which was not confirmed by molecular testing. The remaining 105 patients, 81 males, 24 females, were included in the FXS study.

All 105 patients underwent standardized clinical examination including measurements and evaluation of dysmorphism. Personal and family history were obtained from the parents or legal representative. A positive familial history of ID was defined as the presence of another relative with ID up to the 3rd degree.

During the consultation, we asked the parents to complete a predesigned questionnaire compiling items from the 3 selected screening checklists for FXS, i.e. from Hagerman (Hagerman, Amiri et al. 1991), Maes (Maes, Fryns et al. 2000) and Guruju (Guruju, Lavanya et al. 2009). We used the checklist from Hagerman because it is the most widely used and also because it was the basis for most other, adapted screening lists (Tuncbilek, Alikasifoglu et al. 1999; Guruju, Lavanya et al. 2009). The Maes' checklist takes into account the fact that not all items have the same prediction power. They attributed coefficients to the most predictive items, thus increasing the chance for a fragile X patient to rank at the top. Because of this improvement we also selected this checklist for our study. We also included the checklist from Guruju who included additional physical features and adjusted the threshold compared to the reference checklist from Hagerman.

All items from the 3 checklists were merged into one questionnaire. Each item was accompanied with an additional short description (Addendum 1). One week before the interview, the research questionnaire was sent to parents to make them familiar with the questions and help them prepare their answers. On the day of the clinical examination, we personally administered questions from the questionnaire and scored items. Parents or legal representatives were asked to respond with **YES** when they were certain that the item described in the questionnaire was currently present or had been present in the patient, **NO** when they were convinced that the item had never been observed or **NOT SURE** when they were uncertain. These responses were then translated into scores according to the scoring rules for each of the 3 checklists. Hagerman's checklist has 3 scales, and thus YES was scored

as 2, NOT SURE as 1 and NO as 0. Guruju and Maes were 2 scales scoring systems in which YES received the score 1 whereas NOT SURE and NO received the score 0. When a score is equal or above to the referral scores, referral for molecular genetic testing is indicated. Referral scores are ≥ 10 for Hagerman, ≥ 17 for Maes and ≥ 5 Guruju. We used item frequencies to determine the most prevalent features in our patient cohort and the cumulative scores to assess the sensitivity and specificity of each checklist.

1.12.2 DNA extraction

Venous blood was sampled from a peripheral vein and genomic DNA was extracted by the salt saturation method as previously described (Miller, Dykes et al. 1988). Between 2010 and 2012 DNA extraction was done in the Centre for Human Genetic at Leuven. From 2013 onwards, a DNA extraction facility was implemented at the Institut National de Recherche Biomédicale (INRB) in Kinshasa so that white blood cell pellets or DNA were henceforth prepared at the INRB and then shipped to the Centre for Human Genetics, Leuven, Belgium, for further analysis.

1.12.3 FMR1 CGG repeats testing

To assess the fragment length of the CGG repeats in the promoter region of the FMR1 gene, the target region was amplified during a PCR reaction using the PRC-enhancer kit (Invitrogen) and the FRAXA-A and FRAXA-B primers.

Primer sequences:

FRAXA-A: GAC GGA GGC GCC GCT GCC AGG6FAM

FRAXA-B: GTG GGC TGC GGG CGC TCG AGG

The reaction mix contained 5.6 µl of water, 2 µl of the 10X PCRx Amplification buffer, 0.6 µl of 50 mM $MgSO_4$, 1 µl of dNTPs (4 mM), 8 µl of PCRx Enhancer Solution, 1.5 µl of Primer mix (10 pm/µl) and 0.25 µl of Taq DNA Polymerase from Roche. The cycling comprised an initial denaturation at 95°C for 3 minutes followed by 27 amplification cycles made of short denaturation at 95°C for 15 seconds, annealing at 64°C for 1 minute and elongation at 75°C for 1 minute. The reaction was terminated with a final elongation at 75°C for 7 minutes and cooling at 15°C ∞. PCR product was controlled on 2 % agarose gel with 1 kb size marker. Then, 2 µl of the remaining PCR product was resuspended with an admixture of 20 µl HiDi Formamide (Applied Biosystems) and Rox 500 (Applied Biosystems). Fragment were separated on the on ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA 94404 USA) then analysed with GeneMapper® Software 5 (Life Technologies Ltd, Paisley, UK).

1.12.4 Ethics

The participants were duly informed about the structure and aims of the study. They were informed concerning their right to withdraw from the study. For each participant, parents or legal representatives provided written consent for study participation. We applied an anonymous and non-personal coding system to protect participants' privacy. Our research protocol was approved under the number ESP/CE/008/2015 by the National Ethical Committee of the Public Health School of the University of Kinshasa, Kinshasa, the DR Congo.

1.13 Results

1.13.1 FXS clinical checklists

Using the Hagerman checklist our patients obtained cumulative scores ranging from 2 to 16 with an average of 8.13 ± 3.07. Thirty-nine patients (37.14 %) showed a total score in the referral range (fig. 1).

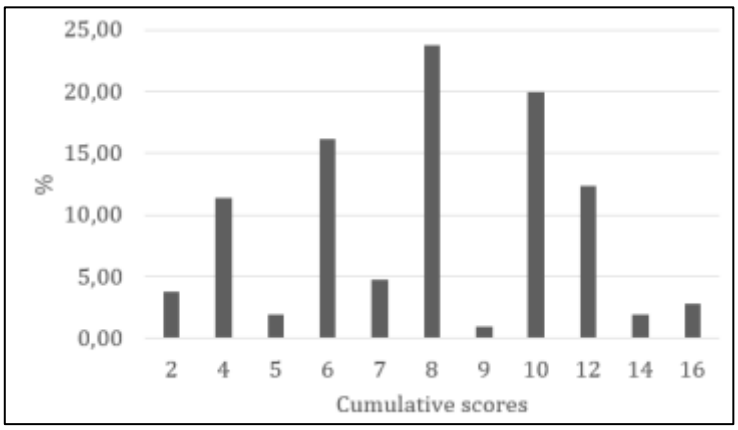

Figure 1. Frequency of cumulative scores with the Hagerman checklist

In addition to the ID present in all patients, behavioural features of attention deficit (62.86 %), hyperactivity (62.86 %) and gaze avoidance (46.67) were especially frequent, compared to dysmorphic features of large and prominent ears (18.09 %) or simian crease (0.95 %) (figure 2).

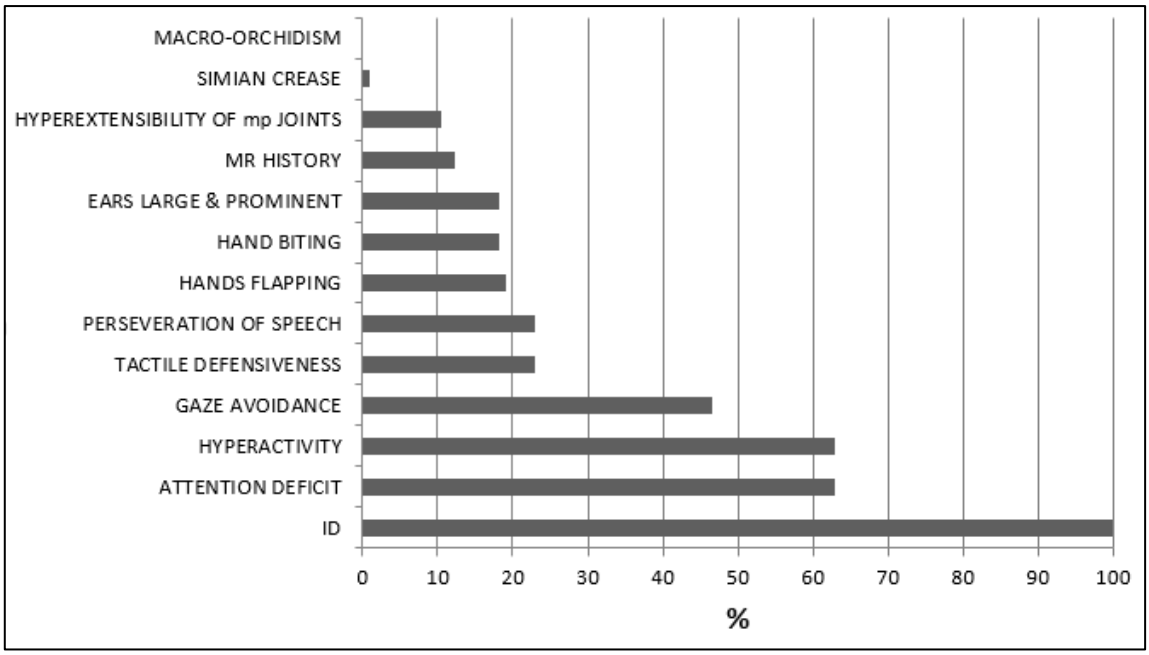

Figure 2. Frequencies for items from Hagerman's checklist

For the Maes' checklist, the cumulative scores ranged between 2 and 27 with an average of 14.30 ± 5.92 (figure 3). A total of 37 patients (35.24 %) had a score within the referral range.

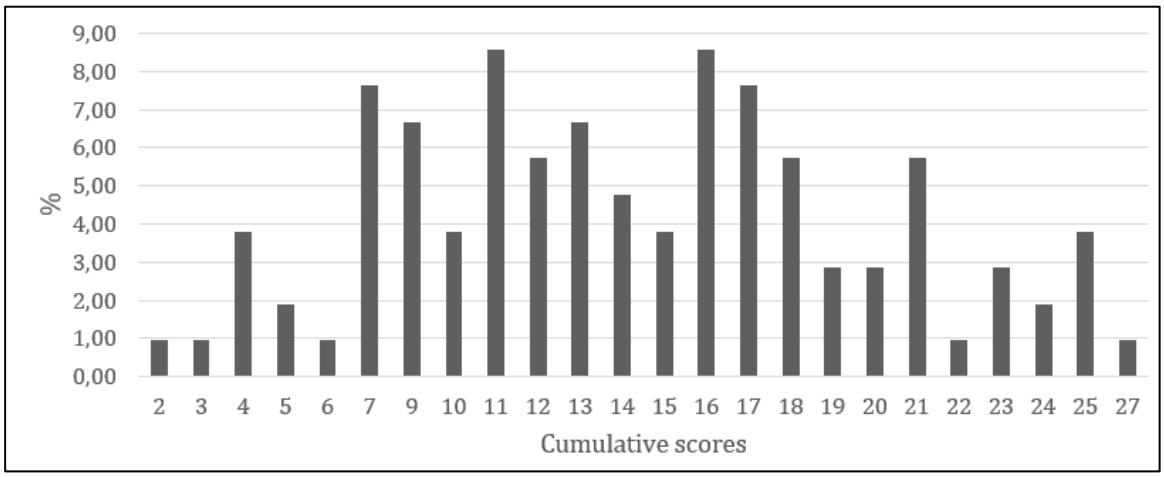

Figure 3. Frequency of the cumulative scores from Maes' checklist

Also in this checklist, behavioural items including autistic-like components such as hypersensitivity to change, limited language, gaze avoidance and stereotypic hand movements, were more frequent compared to dysmorphic features (figure 4).

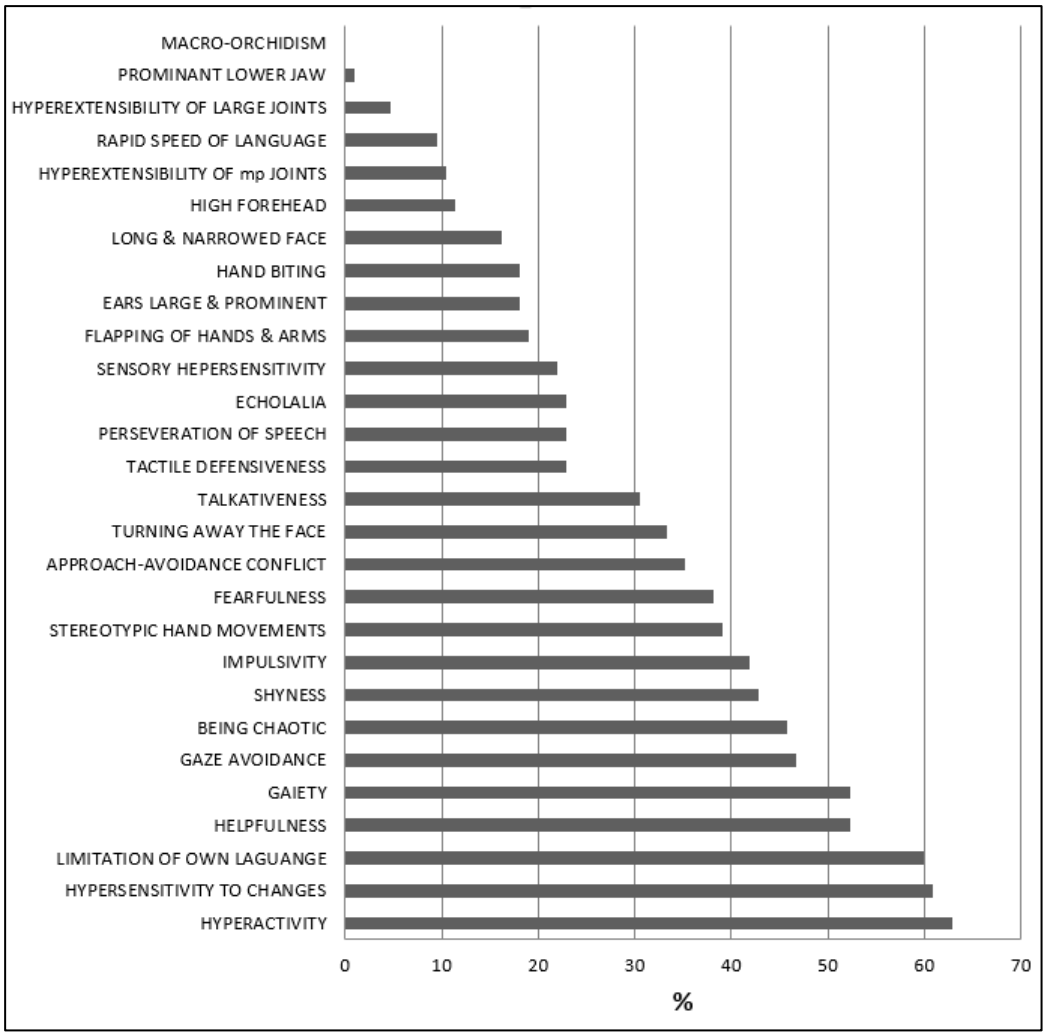

Figure 4. Frequency of items from the Maes' checklist.

On the Guruju's checklist, the cumulative scores ranged from 1 to 10 with an average of 4.24 ±1.75. 46 patients (43.80 %) had a total score ≥5, which means a high probability of FXS (figure 5).

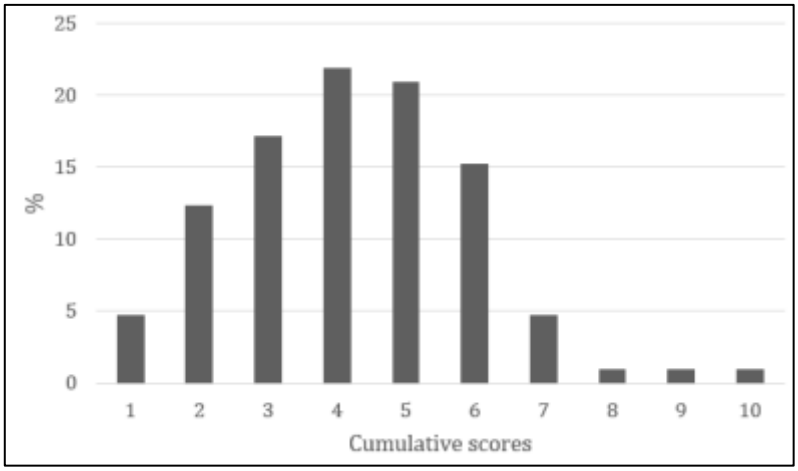

Figure 5. Frequency of the cumulative scores from Guruju's checklist

Also in this checklist, behavioural features were more frequently observed than dysmorphism (fig. 6.).

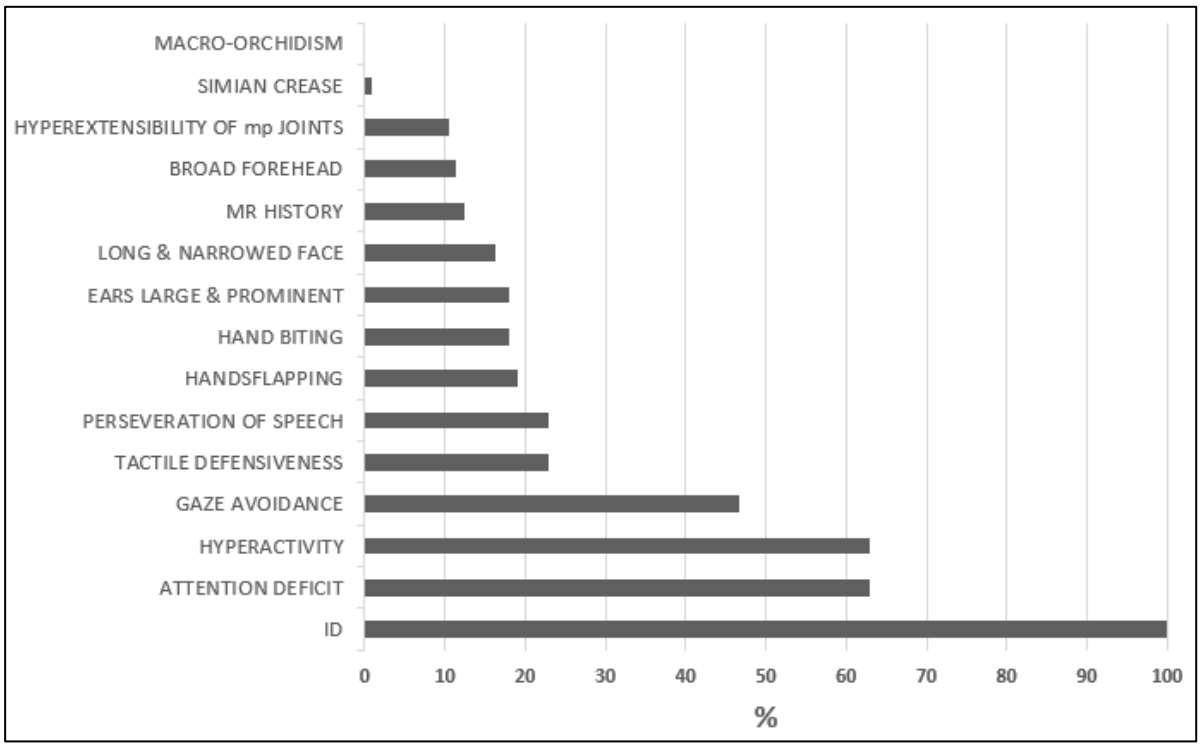

Figure 6. Frequency of items from Guruju's checklist

1.13.2 CGG alleles sizes

We analysed a total of 130 chromosomes including 80 from males and 50 from 25 females. Twenty one different alleles were observed. The average repeat size was 28.55 ± 2.83 (ranges: 17-48) (figure 7). The most frequent allele size was 29 (24.61 %) and 73.84 % of alleles have sizes between 28 and 31 repeats. There was a second and small peak at 21 repeats. No full mutations nor premutations were detected. In 2 male patients an intermediate allele size with 47 and 48 repeats was detected. The 47 repeats allele was inherited from the normal mother who had highly skewed X-chromosome inactivation (92.75 %/7.25 %). DNA of the mother of the patient with 49 repeats was not available.

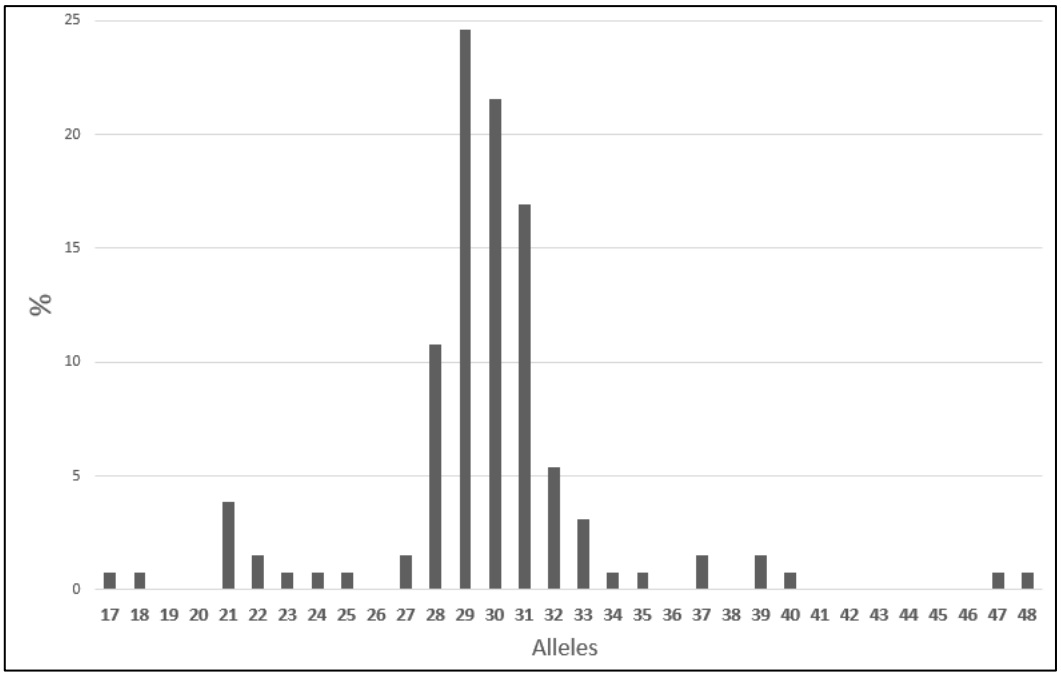

Figure 7. Distribution different CGG alleles

1.13.3 Proportion of behaviour features and sensitivity of checklists

The total score ranged between 2-16 for Hagerman, 2 to 27 for Maes and 1-9 for Guruju. The average number of behavioural items scored positively per patient was 2.55 with Hagerman's and Guruju's tools and 7.79 items with Maes' tool. Conversely, the average number for positive physical features was 0.29 for, 0.295 for Hagerman, 0.57 for Guruju and 0.62 for Maes.

Given the absence of molecularly confirmed Fragile X patients in our cohort, sensitivity could not be calculated for the 3 checklists. However, we calculated the specificity, using the referral thresholds and found 62.86 %, 64.76 % and 56.5 % respectively for Hagerman, Maes and Guruju (table 2).

1.14 Discussion

The Fragile X syndrome is considered to be one of the most common causes of ID worldwide. However, no data exist on the incidence of FXS in Central Africa. This is the first study to evaluate this question in a systematic way in a high risk population of individuals attending schools for special education or specialized clinics for persons with ID.

Among 105 individuals, 80 males and 25 females, not a single case of FXS was diagnosed with molecular testing. This may seem in contradiction with the observations made in South Africa were Goldman et al. reported a prevalence of 6.1 % among black patients in specialised schools and the results of Essop and Krause who identified FXS in 5.2 % in black patients with ID who underwent FMR1 testing in a

period of 25 years in Johannesburg (Goldman, Jenkins et al. 1998; Essop and Krause 2013). However, given the presence of intermediate alleles in our population (2/130 chromosomes), and since intermediate alleles may be unstable, we can anticipate that FXS is very likely to be present in the Congolese population. The absence of positive cases in our study in specialised institutions and hospitals may be explained by certain limitations of this study. One of the limitations is the relatively small size of studied sample. Also, given the limited resources in this country, not all parents can afford paying the specialized institutions and the majority of families are forced to keep their children at home, especially when they have severe behavioural manifestations or disabilities. Consequently, only patients from families with high revenues as well as those with less severe manifestations are encountered in these institutions. This may have resulted in an underrepresentation of FXS cases in this study. Also, FXS patients in DR Congo may present differently and thus be oriented to different specialties such as psychiatry or speech therapist.

In order to increase the chance to identify FXS in Congolese patients, future studies should include a larger cohort of individuals, from different environments (schools, hospitals and a neighbourhood survey), and in collaboration with various medical specialties (psychiatry, neurology, paediatrics, etc). In addition, the recruitment of familial cases, with a pedigree compatible with X-linked inheritance may increase the likelihood of identifying FXS cases.

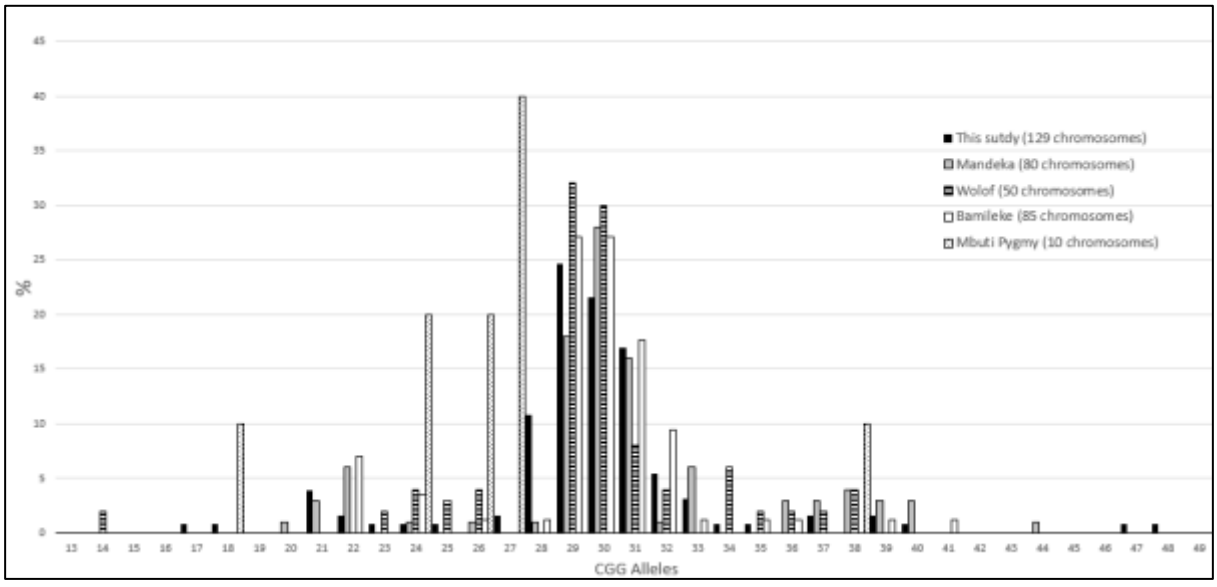

Figure 8. Comparison of CGG distribution to 4 other African groups

We have observed only 21 different alleles among the 129 studied chromosomes (Fig.7). Crawford et al. reported a larger number of alleles in Caucasians (42 alleles) compared to African Americans (37 alleles) (Crawford, Zhang et al. 2000). The number of distinct alleles in the Congolese is similar to those

reported in other African groups (figure 8): 17 for Senegalese Mandeka (Kunst, Zerylnick et al. 1996), 14 in Senegalese Wolof, 13 in Cameroonians Bamileke (Chiurazzi, Destro-Bisol et al. 1996) and 10 for Mbuti Pygmies from DR Congo (Eichler and Nelson 1996).

Alleles in our cohort ranged from 17 to 48 repeats (mean: 28.55 ± 2.83). Interestingly, about 74 % were between 28 and 31 repeats and we detected only 2 alleles shorter than 20 repeats and 2 larger than 45 (Figure 7). This observation is consistent with the prior observation that shorter and larger alleles are rare in African Americans and Africans (Crawford, Zhang et al. 2000; Peprah, Allen et al. 2010). The mean repeat size of 28.55 we observed is similar to the literature reports (Tuncbilek, Alikasifoglu et al. 1999; Tzeng, Cho et al. 1999; Maddalena, Richards et al. 2001; Van Esch 2006; Yim, Jeon et al. 2008; Peprah, Allen et al. 2010). Alleles in our cohort form 2 clusters. A major cluster around the mean, and a minor cluster at 21 repeats. A similar 2 peaks pattern has been previously reported in African and non-African populations (Chiurazzi, Destro-Bisol et al. 1996; Kunst, Zerylnick et al. 1996; Crawford, Zhang et al. 2000; Peprah, Allen et al. 2010). In the majority of populations studied worldwide, the most common allele is either 29 CGG or 30 CGG or an equal frequency of both. Our results tend to corroborate the previously made assumption that 29 was the most common allele in African whereas the 30 was the most frequent in Caucasians (Essop and Krause 2013). In addition, isolated populations such as Japanese, Pygmies (DR Congo) or Mayan (Mexico) are enriched for alleles 27 or 28 repeats (Eichler and Nelson 1996; Otsuka, Sakamoto et al. 2010) associated to a reduced frequency of the 29 and 30 CGG alleles. Although originating from the same country, patients in our study, all from the Bantou population, have different CGG distribution from Mbuti pigmies. Possible founder effects in pigmy group is thought to be at the origin of the altered allele distribution in pigmies (Eichler and Nelson 1996; Otsuka, Sakamoto et al. 2010).

After the results from molecular testing of *FMR1* were available, we evaluated the 3 selected checklists knowing that there was no true positive patient in our cohort. The clinical scoring tools indicate a higher frequency for behavioural characteristics compared to physical features. This may be ascribed to the younger age of patients or to the absence of true fragile X in the cohort. Attention deficit and hyperactivity are the most common items. It may be that parents or tutors tend to overrate these features. It may also represent a natural characteristic of Congolese children consistent with the observation that Congolese children have and advance of about 6 months for motor development but have 6 months delay for adaptive development compared to Caucasians (Tady 2002). Importantly, our study suggests that these behavioural characteristics are so common in non-FXS children with ID, and therefore, incorporating these behavioural features in FXS screening lists will result in low specificities,

at least at thresholds used in the original screening tools. However, the predictive power of each feature can only be determined after identification of fragile X positive cases.

Mean scores recorded in the present study are close to those reported from non-fragile X individuals in original studies (Table 1).

Table 1. Scores from our study compared to the original studies

	Categories	This study			Original study		
		Range	Mean	SD	Range	Mean	SD
Hagerman	FX (-)	2 - 16	8.13	3.07	2 - 23	10.2	NA
	FX (+)	NA	NA	NA	10 - 22	17.3	NA
Maes	FX (-)	2 - 27	14.30	5.92	0 - 24	NA	NA
	FX (+)	NA	NA	NA	16 - 49	NA	NA
Guruju	FX (-)	1 - 9	4.25	1.75	2 - 9	5.44	1.29
	FX (+)	NA	NA	NA	4 - 15	7.05	1.09

Interestingly, if we consider the referral scores, a higher proportion of patients would have been referred for genetic testing in our study, indicating a higher rate of false positives. This is reflected in the much lower specificities of the 3 checklists in our cohort compared to original studies (Table 2). Based on the specificity only, we can anticipate that the 3 checklist have poor performances on Congolese population. An adaptation would be necessary to improve the performance in Congo. Nevertheless, final conclusion can only be drawn in comparison to the specificity when a positive patient will be identified.

Table 2. Specificities in this study versus the original studies

Checklists	Referral threshold	This study	Original studies
Hagerman	≥ 10	62.86 %	87 %
Maes	≥ 17	64.76 %	92.3 %
Guruju	> 5	56.5 %	73.18 %

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CHALLENGES FOR GENETIC STUDIES OF ID IN DR CONGO

1.16 Introduction

Genetic studies of intellectual disability (ID) are almost inexistent in Central Africa, despite the fact that they offer many opportunities to gain insight into the genetics of human disease.

The African population is evolutionary the most ancient and has a unique genetic structure. There is evidence that the mechanisms leading to mutations may differ in Africans compared to other populations (Veltman and Brunner 2012). For instance, the recurrent microdeletion in chromosome 17q21.31 mediated by low copy repeats only occurs in the germline of a parent carrying a 900-kb inversion polymorphism of this region (Koolen, Sharp et al. 2008). This inversion is common in individuals of European ancestry, but rare in Africans (Stefansson, Helgason et al. 2005). The evolutionary history may also explain why certain disorders have a different incidence in the Central African population. The classical example is the high incidence of sickle cell disease in Africans, due to an increased resistance of carriers against malaria infection. Another example is the increased incidence of a 2.7 kb deletion in the P-gene, causing oculocutaneous albinism type 2. Recently, the incidence and/or spectrum of mutations causing common, mostly autosomal dominant monogenetic disorders was found to differ. This was revealed by an approximately 50 % lower incidence of known mutations in a list of actionable genes in African Americans (Dorschner, Amendola et al. 2013; Amendola, Dorschner et al. 2015). Also, the incidence of many autosomal recessive conditions varies wildly, and some conditions are unique to certain populations. This is attributed to evolutionary factors such as consanguinity with founder effects and sometimes natural selection. In certain regions of DR Congo, consanguinity is part of the culture. Examples include Manianga in the province of Bas-Congo and Nande in North-Kivu. Genetic studies in Central Africa offer thus a unique opportunity to identify novel genes or mutations or mutational mechanisms, besides being relevant for the local population.

However, the challenges are enormous.

First, the laboratory infrastructure for genetic testing is almost entirely lacking. Since the onset of this project, I contributed in establishing a DNA extraction facility in the genetic centre in Kinshasa. The first genetic tests are being introduced (karyotyping and diagnosis of sickle cell disease). However, there remains a long way to go before one can envisage routine genetic testing such as microarray-CGH, MLPA or sequencing. In the near future, gene panel analysis or even whole genome sequencing will become a first choice test for the detection of both SNV's and CNV's at an affordable price. Whereas this may seem to overcome the technical challenges, the bioinformatics infrastructure with

fast internet access needed to variant interpretation may be an even larger hurdle to overcome in a country where electricity supply is unpredictable.

Also, clinical genetics faces many challenges. The societal aspects have been discussed in the general introduction. Knowledge of most genetic syndromes is based on Caucasian patients. Knowledge on African patients is mostly limited to African American cases. Since people with a different ethnicity have distinct facial features, it is not unexpected that the dysmorphic features in specific syndromes also differ. Only few studies exist that have explored this issues, none for central African patients. Also, environmental factors may influence the manifestations and clinical course of specific genetic disorders or syndromes. In Central Africa, the high burden of serious infectious diseases, birth trauma and nutritional deficiencies are factors that have to be taken into account during a clinical genetics evaluation.

In this chapter, we explore these different opportunities and challenges, using examples from our study on ID, but equally on other families with other genetic conditions, we observed during our clinical work in Kinshasa.

First, we study differences in facial dysmorphism in African and Caucasian patients. We compare differences in clinical evaluation of facial dysmorphism by African and European clinicians. We also assess differences in facial morphology in African and European Down syndrome patients, using Face2Gene, a recently introduced tool that permits an objective evaluation of facial features.

Next, we describe two instances where the manifestations of a well-known genetic disorder have been modified due to intervening environmental influences. This resulted in an atypical picture and thus delayed diagnosis.

Finally, we describe a familial case of Apert syndrome carrying a unique mutation thus far never described. The overwhelming majority of Apert cases carry the same mutation and we detect, in the first African case ever sequenced a novel mutation. This leaves us wondering whether this is coincidence or related to his central African origin.

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1.17 Detection of facial dysmorphism in central African patients

(Manuscript in preparation)

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1.17.1 Introduction

Facial dysmorphism means that the facial aspect significantly deviates from what is expected. Since many syndromes (genetic or acquired) have a characteristic craniofacial appearance, the recognition of such atypical facial features may contribute to reaching an etiological diagnosis. Facial dysmorphism consists of the presence of multiple minor morphologic (physical) anomalies. Minor physical anomalies in turn may be defined as variant morphological features, are found in less than 4 % of the general population and have no serious medical or cosmetic significance to the individual (Jones 1988; Ambrosio-Gallardo, Cruz-Fuentes et al. 2015). Some of these are qualitative such as the presence of a transverse palmar crease or postaxial polydactyly. Others, like an increased distance between the eyes can be measured and are thus quantitative. For the latter, abnormal is defined as ±2 standard deviations" (SD) from the mean. However, some quantitative features such as anteversion of the nostrils cannot be measured in a routine clinical setting; therefore, their evaluation is usually subjective. Efforts have been undertaken to allow an uniform description of dysmorphism. In 2009, a standardized terminology was proposed to describe various dysmorphic features in different parts of the body (Allanson, Cunniff et al. 2009). Several studies have established a relationship between the number of minor anomalies and the chance of having an additional major malformation (Hennekam 2011). This suggests that minor anomalies may be regarded as the result of a more generalized disturbance of the developmental process and can be used in clinics to suspect a developmental problem. The presence of three or more minor anomalies in a patient can be used as an operational definition of 'dysmorphism'. In practice however, a dysmorphological examination involves a more global or facial gestalt evaluation. Experienced dysmorphologists often recognize a child with a specific syndrome at first sight, based on similarities to other patients with a known syndrome. Subsequently, they search for the specific signs or features associated to this syndrome, to substantiate the supposed diagnosis.

However, the recognition of facial dysmorphism is not easy and is hampered by several factors. The expected, normal facial features depend on the age, sex and ethnic background. It may be influenced by the position during examination (static as in clinical pictures or dynamic when examining the individual 'life') and confounding factors such as strabismus, neurological manifestations or deformations. A further limiting factor in the evaluation of dysmorphism is that this remains largely a subjective evaluation and requires significant experience. Therefore, efforts are ongoing to facilitate a more objective clinical evaluation of dysmorphism. Recent advances in morphometric analysis have explored the possibility to perform an objective evaluation of the facial gestalt from 2D or 3D facial images and reach a reliable syndrome diagnosis. This requires matching the face of a patient with

similar patients in a database of individuals with known syndromes (Hammond, Hutton et al. 2005; Ferry, Steinberg et al. 2014). The primary goal of these computed phenotyping tools is to narrow down the search space by excluding inconsistent diagnosis with great certainty and facilitate further confirmatory testing. One such tool, Face2Gene (http://www.fdna.com/face2gene/) is available online both as a web-based tool and as an application for smart-phones and tablets. Such a user-friendly tool holds great promise of reaching a rapid diagnosis for common genetic syndromes, without the need for expert dysmorphological examination. Especially in low resource countries, where access to laboratory testing is limited, the potential of such a tool is great.

Studying dysmorphism in Central Africa is challenging, because the facial morphology presents obvious differences between African and other populations. First, the incidence of certain minor anomalies differs between populations. For instance, postaxial polydactyly, thick lips vermilions and broad nasal tip are common in African males (Ofodile, Bokhari et al. 1993; Talbert, Kau et al. 2014). Second, the craniofacial presentation of a known syndrome in a patient of African origin may differ from a Caucasian with the same syndrome. This was reported for the Velocardiofacial syndrome (McDonald-McGinn, Minugh-Purvis et al. 2005; Veerapandiyan, Abdul-Rahman et al. 2011), Fragile-X syndrome (Schwartz, Phelan et al. 1988) and fetal alcohol syndrome (Moore, Ward et al. 2007). These variations are thought to hinder the timely diagnosis of manageable diseases and may explain the apparently lower frequency of some disorders in non-Caucasian populations (Veerapandiyan, Abdul-Rahman et al. 2011). Unfortunately, existing reference values for most quantitative traits as well as subjective description of many minor anomalies are largely based on Caucasians but are not available for Africans. This is illustrated by the fact that in the series articles describing the terminology for minor anomalies, pictures from Africans are almost completely lacking.

The aim of the present study is to evaluate the ability to interpret facial morphology in children from Central Africa with ID. First, we evaluated differences in the interpretation of facial gestalt in African children between experienced European and African dysmorphologists. Next, we assessed the performance of the existing computed phenotyping tool Face2Gene, at the current stage of its development, to recognize Down syndrome in Congolese versus Caucasian patients.

1.17.2 Material and Methods

The study is part of an etiological diagnostic study in 127 patients with intellectual disability, recruited in 6 specialized clinics and schools in Kinshasa in the DR Congo. This cohort has been described before (chapter 3).

Part 1. Comparison of scoring of facial (dys)morphology by African and European clinicians

Pictures of all 127 cases (frontal view, and in most cases also a profile) taken during the clinical examination were evaluated by 10 clinicians including 5 from Africa (DR Congo and South Africa), and 5 from Europe (Belgium and United Kingdom). Participants were asked to score the face of each patient, based on the facial gestalt, i.e. without formal and detailed analysis. They could score the face as either normal (score 0), clearly dysmorphic (score 2) or uncertain (score 1).

We hypothesized that African and European physicians would score in the same way (Ho) and used SPSS to calculate *kappa coefficient* for inter-rater agreement (Tang, Hu et al. 2015). The interpretation of the kappa coefficient was made as follows: less than chance agreement when < 0, slight agreement when 0.01–0.20, fair agreement if 0.21–0.40, moderate agreement if 0.41–0.60, substantial agreement when 0.61–0.80 and almost perfect agreement when 0.81–0.99 (Viera and Garrett 2005). We were also interested in potential correlation between the group-score a patient received and the objective number of minor anomalies on the face. We used the Pearson correlation coefficient (r) to calculate the correlation and used the t-test to measure the significance. Interpretation of the Pearson Correlation Coefficient in terms of magnitude of effect sizes was done according to Cohen J (Cohen 1988) as following: “small” if r 0.10-0.30, “medium” when 0.30-0.50 and “large” for r of 0.50 and above.

Part 2. Assessment of FACE2GENE

During the clinical examination, we identified 19 patients with a phenotype suggestive of Down syndrome (DS) among the 127 Congolese patients. This diagnosis was confirmed in all cases by means of standard karyotyping, qPCR or microarray-CGH.

We also obtained facial photographs of 20 Down syndrome patients from Flanders in Belgium (K.D. and M.H.). They matched the 19 DS patients from the DRC for sex and age. In the Congolese Down syndrome cohort, there were 10 boys and 7 girls, and age ranged from 1.86-17 years, mean age was 10.09 ± 4.45 yrs. In the Flemish Down syndrome cohort of 20 cases, there were 10 boys and 10 girls, and age ranged from 2.4 years-14 years, mean age 8 ± 3.23 yrs.

Two of the Congolese DS patients were excluded because their facial photographs did not meet the quality criteria of the Face2Gene program. Thus, facial photographs from 17 Congolese and 20 Flemish were uploaded as 'unknowns' to Face2Gene application without adding any phenotypic description or diagnosis. Following its algorithm, Face2Gene extracts facial gestalt signatures from our patient and compares them to patients with known diagnostics in its database. Then, the top ranking 10 matches are reported, from the most likely to the less likely (Figure 1). Each patient received a score, from 1-10, corresponding to the ranking of a match "Down syndrome" among the 10 matches suggested for that patient by the application. A conservative score of 11 was assigned when "Down syndrome" was not listed within the first 10 matches. The Face2Gene ranks for DS in Congolese and Belgian patients were compared using Wilcoxon test statistics and permutation tests. The Wilcoxon test statistic was calculated using R (version 3.1.1) and compared to 10,000 permutation tests H_a: $\mu_C > \mu_B$). This was repeated 1,000 times to create a p-value interval for decision making. We also calculated the specificity and sensitivity of Face2Gene for Down Syndrome in Congolese and Belgians. We calculated the Accuracy and Precision of Face2Gene. The Accuracy of a measurement system is the degree of closeness of measurements obtained with that measurement system to that quantity's actual (true) value. Conversely, the Precision of a measurement system is the degree to which repeated measurements show the same results under unchanged conditions (http://simple.wikipedia.org/wiki/Accuracy_and_precision).

Figure 1. Illustration of the Face2Gene report. Top panel displays the patient's photographs uploaded to Face2Gene, the green lock indicates that images fulfil quality criteria for the tool. The middle and bottom panel show the 10 matches. For each match, the heating cloud indicates similarities between facial marks identified from the known diagnosis (left) and the patient (right).

1.17.2.1 Ethics issues

The participants were duly informed about the structure and aims of the study. They were informed concerning their right to withdraw from the study. For each participant, parents or legal representatives provided written consent for study participation. We applied an anonymous and non-personal coding system to protect participants' privacy. Our research protocol was approved under the number ESP/CE/008/2015 by the National Ethical Committee of the Public Health School of the University of Kinshasa, Kinshasa, the DR Congo.

1.17.3 Results.

1.17.3.1 Comparison of the scoring by African and European clinicians

African physicians attributed more frequently score 2 (290 times) than Europeans (222 times) and conversely scored less 0 (185 times) than the European colleagues (296 times) (Table 1). We defined the rater's Cumulative Score as the sum of the 127 individual scores given by each rater. Mean cumulative score from the African raters was higher, 148 ± 27.78 (range 110 to 176) compared to Europeans 112.20 ± 27.48 (range 84 to 142).

To assess whether the two groups of physicians had the same perception of dysmorphism in African patients we calculated the inter-rater coefficient of agreement. The kappa-coefficient was 0.29, corresponding to a fair agreement between the 2 groups.

Table 1. Count of times each individual score has been used by each physician and group.

	African Physicians						**European physicians**					
Raters	1	2	3	4	5	Total	A	B	C	D	E	Total
Score 0	58	26	20	42	39	185	80	54	75	48	39	296
Score 1	28	26	49	9	48	160	10	22	17	34	34	117
Score 2	41	75	58	76	40	290	37	51	35	45	54	222
Total	127	127	127	127	127	635	127	127	127	127	127	635

Legend: Columns 1 to 5 correspond to African raters and A to E to Europeans; Rows contain number of times the score in the first column has been use by the rater. For instance the African rater 1 attributed the score 0 to 58 photos and all 5 African physicians used the score 0 a total of 185 times out of 635.

1.17.3.2 Correlation between score and number of minor anomalies on the face

Besides the evaluation of the facial gestalt, a detailed dysmorphic examination was done during the physical examination (A.L, K.D., P.L.), during which all minor facial anomalies were recorded. We counted the number of minor facial features for each individual. We wanted to see whether the gestalt-based scoring for a patient correlated with number of minor anomalies he carried. To derive the correlation for scores from African raters, we tailed the scores from the 5 raters and did the same for the number of minor anomalies. The Pearson correlation coefficient was 0.409 (p=0.000) for African raters and 0.417 (p=0.000) for Europeans, consistent with medium correlation effects in both groups.

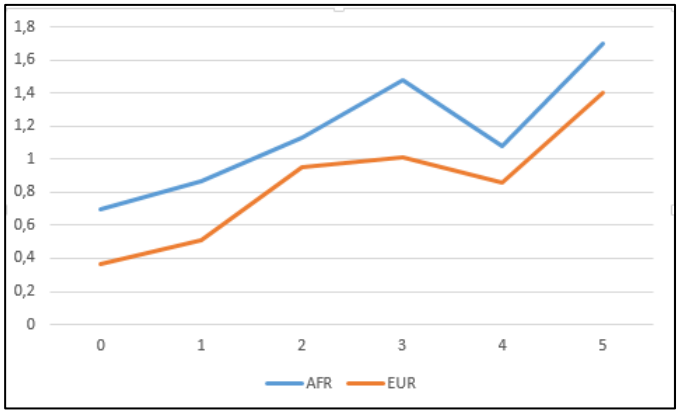

Figure 2. Relation between mean score and number of minor dysmorphism. The mean score shows an increase almost proportionate to number of minor anomalies.

1.17.3.3 Assessment of FACE2GENE for Down syndrome matches

Among the 17 Congolese DS patients, Face2Gene reported matches with DS (i.e. within the first 10 matches) only in 7 (35.29 %). For the 20 Belgian DS patients, a match was found in 16 (80 %). In depth analysis showed that for the 17 Congolese cases, Down syndrome was the first suggested match in 2, ranked within the first 5 matches in 5 and within the first 10 in 6 of them (table 2, figure 2). In the 20 Flemish cases, Down syndrome was the first suggested match in 8, ranked within the first 5 matches in 13 and within the first 10 in 17 of them (table 2, figure 3).

Table 2. Ranking of DS match

	Congolese (n = 17)	Flemish (n = 20)
Within first 10	6	16
Within first 5	5	13
Ranks first	2	8

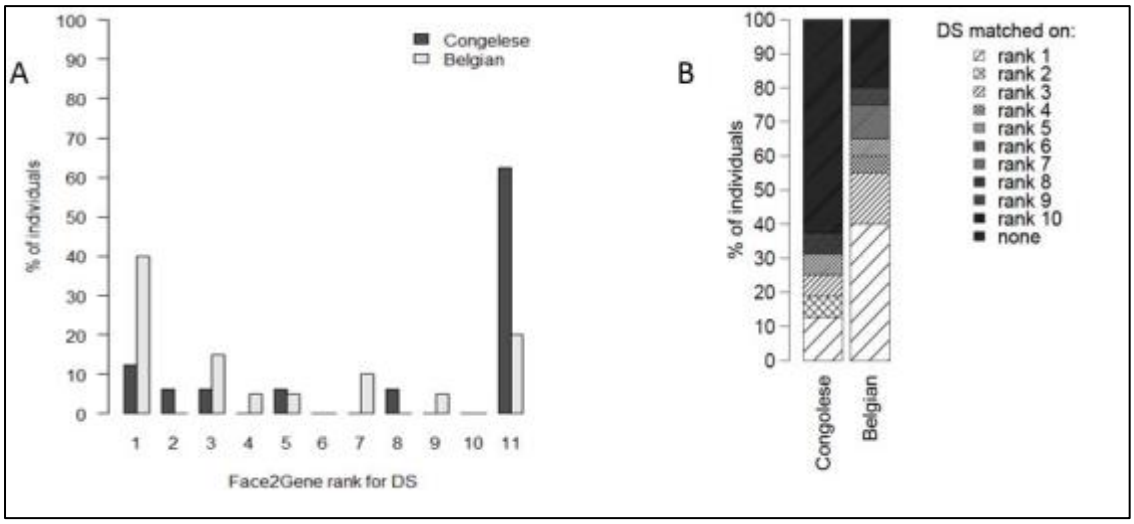

Figure 3. Ranks for DS match. 3A. shows the frequency of DS match in each ethnic group and for each rank. Note the high frequency of Congolese individuals without DS match (rank 11); 3B. Shows the proportion of DS matches at cumulated ranks.

The mean rank in the Congolese patients was 8.29 (Figure 4), which is significantly lower than the mean 4.65 recorded from Europeans ($p = 0.004446 \pm 0.000674$).

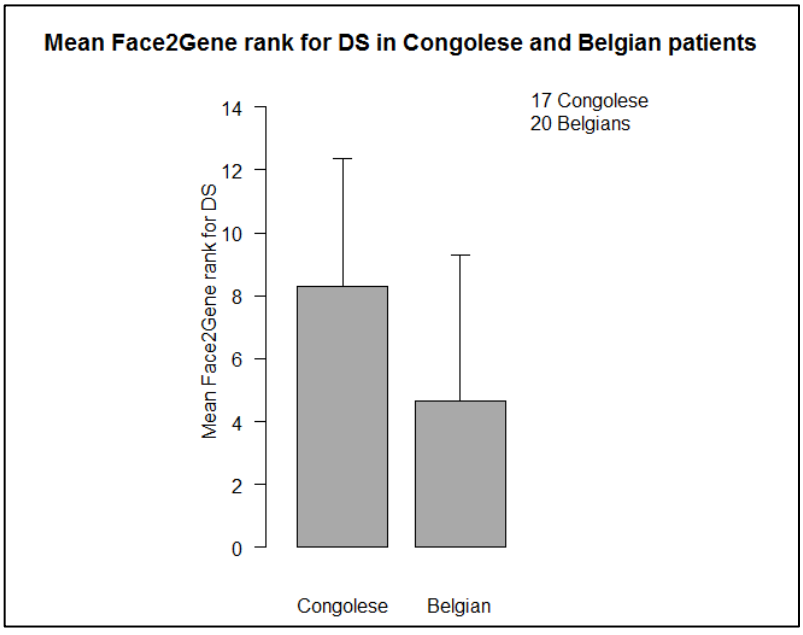

Figure 4. Mean ranking of a match "Down syndrome" in Congolese and European Down syndrome patients

When we considered that DS ranking within the first 10 suggested diagnosis as a positive result, Face2Gene showed an Accuracy of 0.35 in Congolese against 0.8 in Belgians and a Precision of 1 in both groups.

1.17.4 Discussion.

In a first part of this study, we have compared the way African and European clinicians with experience in dysmorphology evaluate facial dysmorphism. In a series of 127 individuals with ID, there was a good correlation between the number of minor facial anomalies, assessed independently during the clinical examination and a “gestalt” evaluation of the facial dysmorphism. This indicates that a “gestalt” evaluation by an experienced clinician is a valuable alternative for a detailed evaluation and counting the number of minor anomalies. The higher the number of minor facial anomalies, the more likely a person was regarded as dysmorphic, which is not unexpected. Of interest, on average, European clinicians were less likely to score an individual as dysmorphic compared to the African clinicians, regardless of the number of minor facial anomalies. We have no clear explanation for this observation, but this variable may need to be taken into account in dysmorphology studies in different ethnicities. It would be of interest to perform the inverse experiment, i.e. scoring faces of Caucasian children with ID by both groups, to see whether the same differences in scoring are made.

These results also indicate that there is an important subjective aspect in the evaluation of (facial) dysmorphism. For this reason, there is interest in tools to obtain an objective evaluation or even a possible syndrome diagnosis. Online applications that can establish a syndrome diagnosis based on a facial picture of a person are becoming a reality. Such systems offer the potential for a universal, rapid and cheap syndrome diagnosis. The performance of such systems is obviously a critical factor. We tested the Face2Gene tool, because we wished to compare its performance in Europeans and Africans.

Our data indicate that the system has a high precision in both groups. However, the accuracy in the Caucasian cohort was much higher compared to the African cohort. This is interesting, since it confirms that there are differences in facial appearance of Caucasian versus African Down syndrome patients. The most likely explanation why Face2gene is underperforming in Congolese Down syndrome is that the tool is trained mostly with Caucasian cases. We therefore anticipate that the performance will improve when the system is trained with more Down syndrome cases from Central Africa. A collaborative effort to test this hypothesis is ongoing. Likewise, we expect that the efficiency of Face2Gene might improve if an increasing number of cases with a known diagnosis are uploaded.

The current experiment suffers from a number of limitations. First, the number of cases included was limited. Also the Face2Gene application is an emerging tool and the number of training cases included might still be insufficient to permit an optimal performance. Most importantly, from a clinical point of view, the cases we tested were young children and adolescents, but no neonates. An early, neonatal diagnosis of Down syndrome is important for correct counselling and adequate follow-up. Given the

changing facial phenotype with ageing, more studies are needed to evaluate the performance of Face2Gene with neonates with Down syndrome.

CONFLICT OF INTEREST

None

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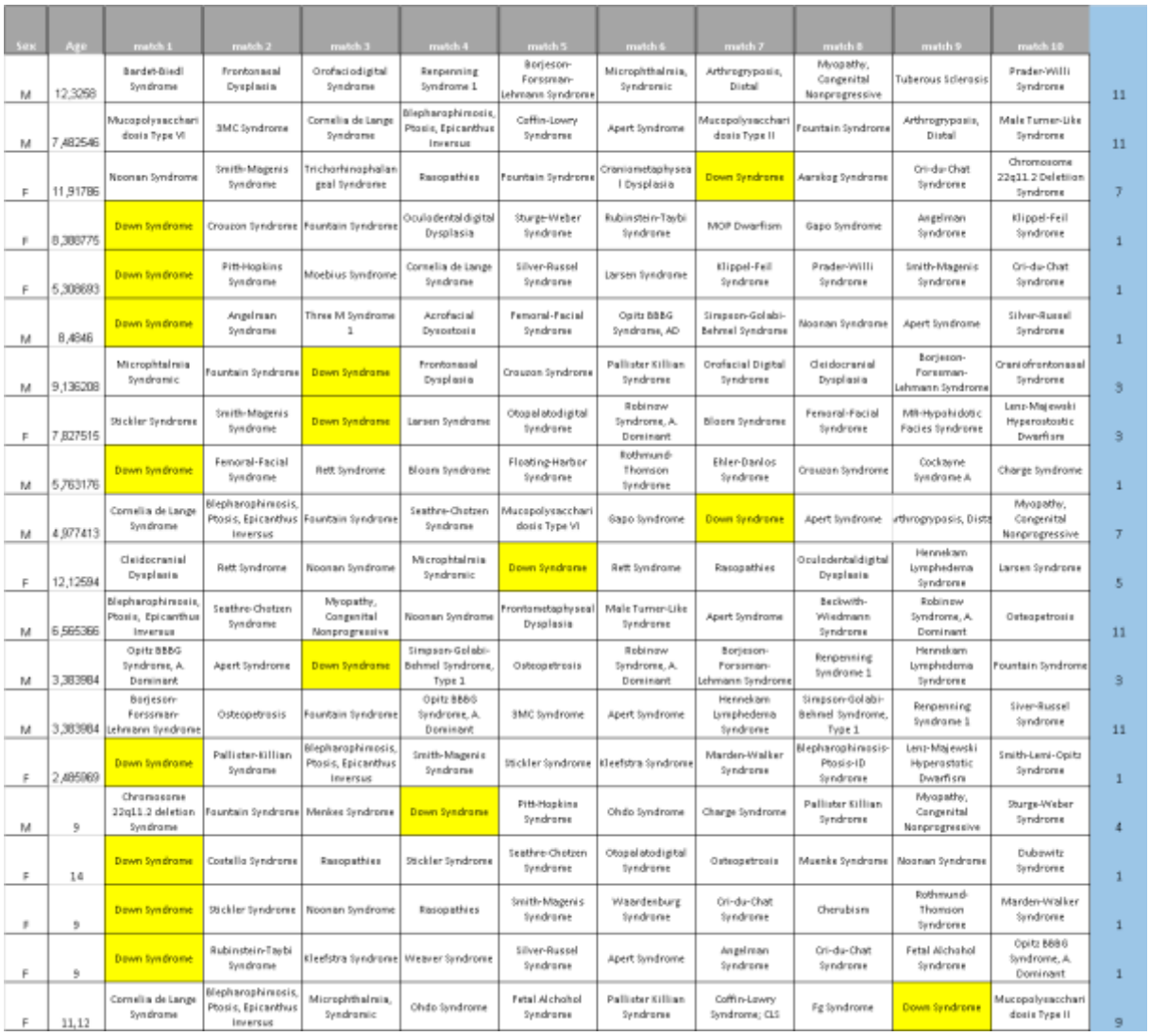
Supplemental figures

Figure 5. Syndrome matches by Face2Gene in Congolese Down syndrome cases

Sex	Age	match 1	match 2	match 3	match 4	match 5	match 6	match 7	match 8	match 9	match 10	RANK
F	1,86	Lipodystrophy, Congenital Generealized	Blepharocheilodontic Syndrome	Spondyloepimetaphyseal Dysplasia	Cherubism	Noonan Syndrome	Costello Syndrome	MOP Dwarfism	Femoral-Facial Syndrome	Kabuki Syndrome	Waardenburg Syndrome	11
M	4,21	Lipodystrophy, Congenital Generealized	Mucopolysaccharidosis Type VI	Cri-Du-Chat Syndrome	Hemifacial Microsomia	Waardenburg Syndrome	Cherubism	MOP Dwarfism	Weaver Syndrome	Lenz-Majewski Hyperostatic Dwarfism	Costello Syndrome	11
M	4,52	Femoral-Facial Syndrome	Lipodystrophy, Congenital Generealized	Hutchinson-Gilford Progeria Syndrome	Treacher Collins Syndrome	Fountain Syndrome	Rothmund-thomson Syndrome	Gapo Syndrome	Ectodermal Dysp. Hypohidrotic, X-Linked	Osteopetrosis	Cutis Laxa	11
M	6,88	Kabuki Syndrome	3MC Syndrome	Cherubism	Baraitser-Winter Syndrome 1	Trichorhinophalangeal Syndrome, Type III	Lenz-Majewski Hyperostatic Dwarfism	Blepharophimosis-Ptosis-Intellectual Disability	Noonan Syndrome	Acrofacial Dysostosis	Ectodermal Dysp. Hypohidrotic, X-Linked	11
M	7,00	Ectodermal Dysp. Hypohidrotic, X-Linked	3MC Syndrome	Gapo Syndrome	Johnson Neuroectodermal Syndrome	Weaver Syndrome	Melnick-Needles Syndrome	Waardenburg Syndrome	Lipodystrophy, Congenital Generealized	Smith-Lemi-Opitz Syndrome	Ohdo Syndrome	11
M	7,70	Lipodystrophy, Congenital Generealized	Down Syndrome	Brachiooculofacial Syndrome	Waardenburg Syndrome	Seathre-Chotzen Syndrome	Mucopolysaccharidosis	Fountain Syndrome	3MC Syndrome	Multiple Pterygium Syndrome	Cockayne Syndrome A	2
F	8,32	Cutis Laxa	Lipodystrophy, Congenital Generealized	Brachiooculofacial Syndrome	3MC Syndrome	Fountain Syndrome	Mucopolysaccharidosis Type VI	Otopalatodigital Syndrome	Mucopolysaccharidosis Type II	Frontometaphyseal Dysplasia	Mucopolysaccharidosis	11
F	9,51	Aarskog Syndrome	3MC Syndrome	Multiple Pterygium Syndrome	Lenz-Majewski Hyperostatic Dwarfism	Down Syndrome	Noonan Syndrome	Osteopetrosis	Gapo Syndrome	Cherubism	Fetal Alchohol Syndrome	5
F	9,93	Lipodystrophy, Congenital Generealized	Hutchinson-Gilford Progeria Syndrome	Osteopetrosis	Cherubism	Waardenburg Syndrome	Blepharocheilodontic Syndrome	Otopalatodigital Syndrome	Down Syndrome	Hurler Syndrome	Mucopolysaccharidosis	8
F	11,03	3MC Syndrome	Brachiooculofacial Syndrome	Melnick-Needles Syndrome	Ectodermal Dysp. Hypohidrotic, X-Linked	Waardenburg Syndrome	KBG Syndrome	Seathre-Chotzen Syndrome	Silver-Russel Syndrome	Rasopathies	Costello Syndrome	11
M	12,52	Cherubism	Ectodermal Dysp. Hypohidrotic, X-Linked	Down Syndrome	Waardenburg Syndrome	Smith-Lemi-Opitz Syndrome	Coffin-Lowry Syndrome	Costello Syndrome	Lenz-Majewski Hyperostatic Dwarfism	Chromosome 15q11-q13 Duplication	Mucopolysaccharidosis Type II	3
F	12,73	Down Syndrome	Cherubism	Lipodystrophy, Congenital Generealized	Treacher Collins Syndrome	Aarskog Syndrome	Ectodermal Dysp. Hypohidrotic, X-Linked	Waardenburg Syndrome	Lenz-Majewski Hyperostatic Dwarfism	Gapo Syndrome	Frontonasal Dysplasia	1
M	13,38	Lipodystrophy, Congenital Generealized	Sturge-Weber Syndrome	Otopalatodigital Syndrome	Mucopolysaccharidosis Type VI	Holt-Oram Syndrome	Trichorhinophalangeal Syndrome, Type II	Gapo Syndrome	Waardenburg Syndrome	Blepharophimosis-Ptosis-Intellectual Disability	Hutchinson-Gilford Progeria Syndrome	11
F	13,73	Down Syndrome	Johnson Neuroectodermal Syndrome	Gapo Syndrome	Ectodermal Dysp. Hypohidrotic, X-Linked	Coffin-Lowry Syndrome	Cutis Laxa	Fountain Syndrome	Moebius Syndrome	Mucopolysaccharidosis Type VI	Chondrodysplasia Punctata	1
M	13,98	Waardenburg Syndrome	Otopalatodigital Syndrome	Stickler Syndrome	Renpenning Syndrome 1	Lipodystrophy, Congenital Generealized	3MC Syndrome	Trichorhinophalangeal Syndrome, Type II	Multiple Pterygium Syndrome	Holt-Oram Syndrome	Rasopathies	11
M	17,08	Ectodermal Dysp. Hypohidrotic, X-Linked	Cherubism	Treacher Collins Syndrome	Seathre-Chotzen Syndrome	Lenz-Majewski Hyperostatic Dwarfism	Moebius Syndrome	Waardenburg Syndrome	Hemifacial Microsomia Syndrome	Stickler Syndrome	KBG Syndrome	11
M	17,3	3MC Syndrome	aardenburg Syndro	Gapo Syndrome	Pfeiffer Syndrome	Trichorhinophalangeal Syndrome	MOP Dwarfism	Femoral-Facial Syndrome	Aarskog Syndrome	Catel-Mantzke Syndrome	Brachiooculofacial Syndrome	11

Figure 6. Syndrome matches by Face2Gene in Flemish Down syndrome cases



Sex	Age	match 1	match 2	match 3	match 4	match 5	match 6	match 7	match 8	match 9	match 10	
M	12,3268	Bardet-Biedl Syndrome	Frontonasal Dysplasia	Orofaciodigital Syndrome	Renpenning Syndrome 1	Borjeson-Forssman-Lehmann Syndrome	Microphthalmia, Syndromic	Arthrogryposis, Distal	Myopathy, Congenital Nonprogressive	Tuberous Sclerosis	Prader-Willi Syndrome	11
M	7,482546	Mucopolysaccharidosis Type VI	3MC Syndrome	Cornelia de Lange Syndrome	Blepharophimosis, Ptosis, Epicanthus Inversus	Coffin-Lowry Syndrome	Apert Syndrome	Mucopolysaccharidosis Type II	Fountain Syndrome	Arthrogryposis, Distal	Male Turner-Like Syndrome	11
F	11,91786	Noonan Syndrome	Smith-Magenis Syndrome	Trichorhinophalangeal Syndrome	Rasopathies	Fountain Syndrome	Craniometaphyseal Dysplasia	Down Syndrome	Aarskog Syndrome	Cri-du-Chat Syndrome	Chromosome 22q11.2 Deletion Syndrome	7
F	8,388775	Down Syndrome	Crouzon Syndrome	Fountain Syndrome	Oculodentaldigital Dysplasia	Sturge-Weber Syndrome	Rubinstein-Taybi Syndrome	MOP Dwarfism	Gapo Syndrome	Angelman Syndrome	Klippel-Feil Syndrome	1
F	5,308693	Down Syndrome	Pitt-Hopkins Syndrome	Moebius Syndrome	Cornelia de Lange Syndrome	Silver-Russel Syndrome	Larsen Syndrome	Klippel-Feil Syndrome	Prader-Willi Syndrome	Smith-Magenis Syndrome	Cri-du-Chat Syndrome	1
M	8,4646	Down Syndrome	Angelman Syndrome	Three M Syndrome 1	Acrofacial Dysostosis	Femoral-Facial Syndrome	Opitz BBBG Syndrome, AD	Simpson-Golabi-Behmel Syndrome	Noonan Syndrome	Apert Syndrome	Silver-Russel Syndrome	1
M	9,136208	Microphtalmia Syndromic	Fountain Syndrome	Down Syndrome	Frontonasal Dysplasia	Crouzon Syndrome	Pallister Killian Syndrome	Orofacial Digital Syndrome	Cleidocranial Dysplasia	Borjeson-Forssman-Lehmann Syndrome	Craniofrontonasal Syndrome	3
F	7,827515	Stickler Syndrome	Smith-Magenis Syndrome	Down Syndrome	Larsen Syndrome	Otopalatodigital Syndrome	Robinow Syndrome, A. Dominant	Bloom Syndrome	Femoral-Facial Syndrome	MR-Hypahidotic Facies Syndrome	Lenz-Majewski Hyperostostic Dwarfism	3
M	5,763176	Down Syndrome	Femoral-Facial Syndrome	Rett Syndrome	Bloom Syndrome	Floating-Harbor Syndrome	Rothmund-Thomson Syndrome	Ehler-Danlos Syndrome	Crouzon Syndrome	Cockayne Syndrome A	Charge Syndrome	1
M	4,977413	Cornelia de Lange Syndrome	Blepharophimosis, Ptosis, Epicanthus Inversus	Fountain Syndrome	Seathre-Chotzen Syndrome	Mucopolysaccharidosis Type VI	Gapo Syndrome	Down Syndrome	Apert Syndrome	Arthrogryposis, Dist	Myopathy, Congenital Nonprogressive	7
F	12,12594	Cleidocranial Dysplasia	Rett Syndrome	Noonan Syndrome	Microphtalmia Syndromic	Down Syndrome	Rett Syndrome	Rasopathies	Oculodentaldigital Dysplasia	Hennekam Lymphedema Syndrome	Larsen Syndrome	5
M	6,565366	Blepharophimosis, Ptosis, Epicanthus Inversus	Seathre-Chotzen Syndrome	Myopathy, Congenital Nonprogressive	Noonan Syndrome	Frontometaphyseal Dysplasia	Male Turner-Like Syndrome	Apert Syndrome	Beckwith-Wiedmann Syndrome	Robinow Syndrome, A. Dominant	Osteopetrosis	11
M	3,383984	Opitz BBBG Syndrome, A. Dominant	Apert Syndrome	Down Syndrome	Simpson-Golabi-Behmel Syndrome, Type 1	Osteopetrosis	Robinow Syndrome, A. Dominant	Borjeson-Forssman-Lehmann Syndrome	Renpenning Syndrome 1	Hennekam Lymphedema Syndrome	Fountain Syndrome	3
M	3,383984	Borjeson-Forssman-Lehmann Syndrome	Osteopetrosis	Fountain Syndrome	Opitz BBBG Syndrome, A. Dominant	3MC Syndrome	Apert Syndrome	Hennekam Lymphedema Syndrome	Simpson-Golabi-Behmel Syndrome, Type 1	Renpenning Syndrome 1	Siver-Russel Syndrome	11
F	2,495069	Down Syndrome	Pallister-Killian Syndrome	Blepharophimosis, Ptosis, Epicanthus Inversus	Smith-Magenis Syndrome	Stickler Syndrome	Kleefstra Syndrome	Marden-Walker Syndrome	Blepharophimosis-Ptosis-ID Syndrome	Lenz-Majewski Hyperostatic Dwarfism	Smith-Lemi-Opitz Syndrome	1
M	9	Chromosome 22q11.2 deletion Syndrome	Fountain Syndrome	Menkes Syndrome	Down Syndrome	Pitt-Hopkins Syndrome	Ohdo Syndrome	Charge Syndrome	Pallister Killian Syndrome	Myopathy, Congenital Nonprogressive	Sturge-Weber Syndrome	4
F	14	Down Syndrome	Costello Syndrome	Rasopathies	Stickler Syndrome	Seathre-Chotzen Syndrome	Otopalatodigital Syndrome	Osteopetrosis	Muenke Syndrome	Noonan Syndrome	Dubowitz Syndrome	1
F	9	Down Syndrome	Stickler Syndrome	Noonan Syndrome	Rasopathies	Smith-Magenis Syndrome	Waardenburg Syndrome	Cri-du-Chat Syndrome	Cherubism	Rothmund-Thomson Syndrome	Marden-Walker Syndrome	1
F	9	Down Syndrome	Rubinstein-Taybi Syndrome	Kleefstra Syndrome	Weaver Syndrome	Silver-Russel Syndrome	Apert Syndrome	Angelman Syndrome	Cri-du-Chat Syndrome	Fetal Alchohol Syndrome	Opitz BBBG Syndrome, A. Dominant	1
F	11,12	Cornelia de Lange Syndrome	Blepharophimosis, Ptosis, Epicanthus Inversus	Microphthalmia, Syndromic	Ohdo Syndrome	Fetal Alchohol Syndrome	Pallister Killian Syndrome	Coffin-Lowry Syndrome; CLS	Fg Syndrome	Down Syndrome	Mucopolysaccharidosis Type II	9

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1.18 Williams-Beuren Syndrome: Pitfalls for diagnosis in limited resources setting

(manuscript under review, Clinical Case Report)

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Short title: **WILLIAMS BEUREN SYNDROME IN A CONGOLESE PATIENT**

KEY CLINICAL MESSAGE

Patients with Williams-Beuren Syndrome can be recognized clinically, given the characteristic dysmorphism, intellectual disability and behaviour. We present a Congolese boy who was diagnosed only when he was 8.2 years old despite the typical WBS facial characteristics. His intelligence and behaviour profile were influenced by a meningitis and coma at the age of 2 years, resulting in a profound intellectual disability and atypical behaviour. The present report supports the notion that infections constitute a major pitfall for genetic diagnosis and should warn clinicians that a genetic disease may be masked by superimposed infectious diseases and brain damage.

Keywords: Williams Syndrome, phenotype, Central Africa, Democratic Republic of Congo.

1.18.1 Introduction

Williams-Beuren Syndrome (WBS) is one of the most common recurrent microdeletion syndromes, with an estimated incidence varying from 1 in 7500 to 1 in 20.000 births (Morris and Mervis 2000; Stromme, Bjornstad et al. 2002). Patients can be recognized clinically, given their characteristic features, encompassing congenital heart defect, typically supravalvular aortic stenosis (SVAS) and/or peripheral pulmonary stenosis, short stature/growth retardation, recognizable facial dysmorphism, developmental delay and intellectual disability, distinct behavioural phenotype, and, in some instances, hypercalcemia. Facial dysmorphism includes a wide mouth, short nose with bulbous or upturned nasal tip, long philtrum, thick everted vermillion of the lips, bitemporal narrowing, periorbital fullness, full cheeks and a small jaw and the characteristic stellate pattern of the iris (Merla, Brunetti-Pierri et al. 2010; Patil, Madhusudhan et al. 2012). The cognitive profile in WBS ranges from mild to severe ID (Martens, Wilson et al. 2008; Merla, Brunetti-Pierri et al. 2010; Mervis and John 2010). Although acquisition of language is often delayed in WBS, they later develop good language skills and become talkative (Pegoraro, Steiner et al. 2014; Tekendo-Ngongang, Dahoun et al. 2014). Behavioural issues in WBS comprise anxiety, hyperacousis, attention problems, hypersociability or friendly behaviour, and strong affinity and interest to music (Doyle, Bellugi et al. 2004; Ng, Lai et al. 2013; Pegoraro, Steiner et al. 2014). Recognition of the typical dysmorphism and characteristic behaviour lead to a clinical diagnosis.

Some cases are more difficult to diagnose, since they present with atypical features (Sakhuja, Whyte et al. 2015), or because they have a different ethnicity where the features may be different from those observed in Caucasians. For instance, African patients with WBS from Cameroon have been reported with incomplete WBS facial dysmorphism (Tekendo-Ngongang, Dahoun et al. 2014). Finally, cases with

a deletion smaller or larger than the recurrent 1.5 Mb 7q11.23 microdeletion may also present with a milder or more severe phenotype (Fusco, Micale et al. 2014).

We here report a WBS patient from Central Africa carrying the recurrent WBS microdeletion who presented with a typical facial dysmorphism but more severe intellectual disability and atypical behavioural phenotype due to a confounding factor, i.e. meningitis with coma supposedly resulting in brain damage.

1.18.2 Case description

An 8.2-year-old Congolese boy was referred by his paediatrician to the genetic clinic at the University Hospitals of the University of Kinshasa, DR Congo, for evaluation of intellectual disability, allegedly resulting from an episode of meningitis and coma. He was born from unrelated, healthy parents after an uneventful pregnancy and delivery. At the age of 2 years, he wasn't able to stand-up without support or build simple sentences although he could call names of his relatives. Four months later, he suffered from meningitis and remained in coma for 2 days. After recovery, he presented severe intellectual disability and complete loss of intelligible speech. His behaviour had changed, including hyperactivity, irritability, hypersensitivity to noise and episodes of aggressiveness against strangers. In contrast, he was friendly towards familiar persons and demonstrated a strong attraction to music and musical sounds. He was diagnosed with sequel from meningitis and was followed by a neurologist until the age of 8 years, when he was referred for a genetic evaluation.

At that time, he had severe global growth delay with microcephaly (OFC 47 cm; -3.82 SD), short stature (length 109 cm; -3.43 SD) and low weight (17 kg; <-3 SD). Family history revealed that two siblings of the patient (Figure 1, panel A) died from infectious diseases. There were two other males with ID in the pedigree, related through their mothers. Unfortunately, these were not available for evaluation. Patient's phenotype consisted of a triangular face with low set ears, long philtrum, flat nasal bridge, bulbous nasal tip, anteverted nostrils, pronounced nasolabial folds, large mouth, thick and everted vermillion of the upper and lower lips and a small jaw (Figure 1, panel B). He also had widely spaced nipples, a small penis, bilateral cryptorchidism and deep palmar creases. Cardiac ultrasound revealed bicuspid aorta with aortic insufficiency.

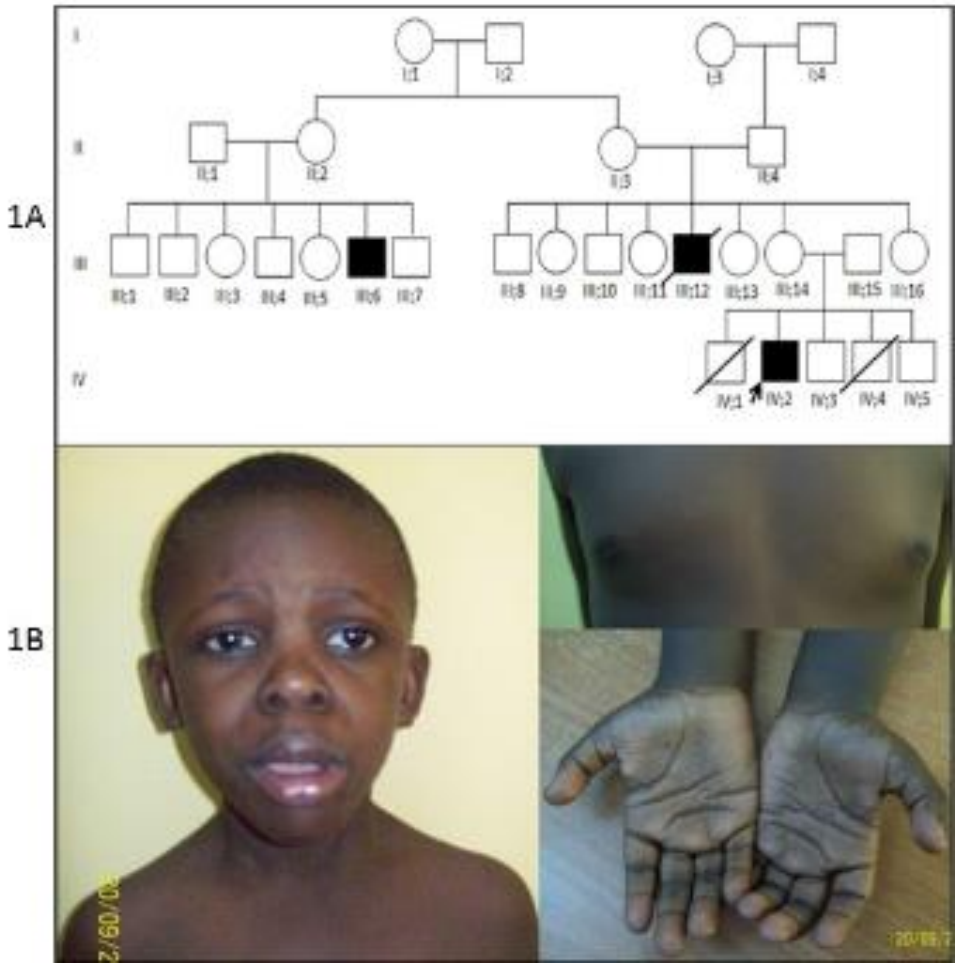

FIG 1. Pedigree and dysmorphic features

Legend: 1A. Pedigree showing 3 males with ID, consistent with the X-linked inheritance; 1B. Clinical features. Note triangular face with low set ears, long philtrum, flat nasal bridge, bulbous nasal tip, anteverted nostrils, pronounced nasolabial folds, large mouth, thick and everted vermillion of the upper and lower lips and a small jaw, widely spaced nipples and deep palmar creases.

Although the pedigree was consistent with X-linked ID, a clinical diagnosis of WBS was retained.

Fluorescent in situ hybridization (FISH) was performed on nuclei from peripheral white blood cells using the commercially available Vysis LSI ELN Kit (Vysis, Abbott Laboratories. Abbott Park, Illinois, U.S.A.). A total of 100 nuclei were examined and a single ELN was observed in all of them, whereas the control probe always showed 2 signals. This confirmed the diagnosis of WBS (Figure 2A). Microarray-CGH was performed using a stripped CytoSure™ ISCA v2 array 8x60k format (OGT, Oxford UK) slide, the CytoSure™ Genomic DNA Labelling Kits (Oxford, UK) and the Oligo aCGH & ChIP-on-Chip Hybridization Kits (Agilent, Santa Clara, CA, 95051 United States) following manufacturers' procedures. This revealed the presence of a recurrent 1.57 Mb deletion arr [hg19]7q11.23 (72,634,874-74,203,685)x1 (Figure 2B).

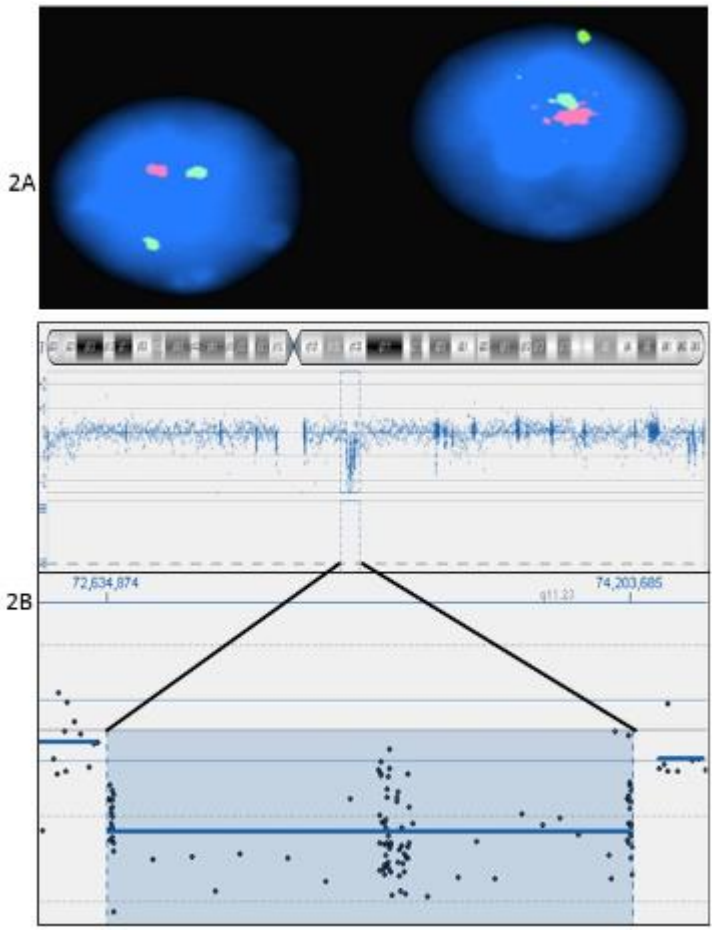

FIG 2. FISH and Array CGH ideograms

Legend: 2A.Two interphase nuclei showing a single signal for the Elastin probe (red) and 2 signals for the control probe (green); 2B. Chromosome icon and the zoom-in on the deleted region.

1.18.3 Discussion

We report on a central African patient with WBS, diagnosed at age 7 years. Mean age of diagnosis for WBS in Western countries is 3.5 years (Huang, Sadler et al. 2002; Ferrero, Biamino et al. 2007). The facial appearance in our patient was typical for WBS. When we uploaded his facial photograph to Fage2Gene tool without adding any further description, the WBS match ranked 5th based only on the gestalt.

Several factors may have contributed to a delayed diagnosis. First, the facial dysmorphism in WBS evolves over age and may be discrete in infancy and early childhood (Huang, Sadler et al. 2002; Ferrero, Biamino et al. 2007; Patil, Madhusudhan et al. 2012). Second, in patients of African origin, the phenotype for several known genetic disorders shows differences from the typical "gestalt" as defined mostly in Caucasian patients (Tekendo-Ngongang et al., 2014).

Examples include the Fragile X syndrome (Schwartz, Phelan et al. 1988; McDonald-McGinn, Minugh-Purvis et al. 2005; Tekendo-Ngongang, Dahoun et al. 2014). Also, our patient had no SVAS, one of the most characteristic features often leading to the diagnosis (Eronen, Peippo et al. 2002; Ferrero, Biamino et al. 2007). Finally, the clinical course of the patient was complicated by a superimposed meningitis at the age of 2 years. As the deletion in our patient corresponds to the classical WBS microdeletion, we assume that the severe speech deficit and profound intellectual disability in the patient are more likely a complication of the meningitis and coma. It is thus likely that an acquired condition modified the clinical course of the patient toward the more severe and atypical phenotype for WBS causing the delay in diagnosis. Such overlapping of genetic disease with an infectious disease is expected to be frequent in the developing world, where infections represent the major medical problem. As previously reported, infections are major cause for misdiagnosis of genetic diseases in developing countries (Lumaka, Mubungu et al. 2012). The present report supports the assumption that infection constitutes a major pitfall for genetic diagnosis and should warn clinicians working in developing world setting that a genetic disease may be masked by a intercurrent infectious disease.

To date, only one report exists concerning WBS in Central Africa (Tekendo-Ngongang, Dahoun et al. 2014). Given the incidence of 1/7500 for WBS (Stromme, Bjornstad et al. 2002), this suggests that most cases remain undiagnosed. This is most likely due to a lack of knowledge and training in the field of dysmorphology and lack of access to genetic facilities in that part of the world.

CONFLICT OF INTEREST

None

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1.19 X-linked Adrenal Hypoplasia Congenita: a novel *DAX1* missense mutation and challenges for clinical diagnosis in Africa.

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1.19.1 Introduction

Adrenal Hypoplasia Congenita (AHC) is a failure in development of the definitive adult adrenal cortex (Phelan and McCabe 2001). This rare disease affects about 1 in 12,500 live births (Laverty, Fortune et al. 1973) and can be inherited as an autosomal recessive or X-linked disease (Ostermann, Salvi et al. 2006; Shaikh, Boyes et al. 2008).

The X-linked AHC has variable clinical presentations (Merke, Tajima et al. 1999; Achermann, Meeks et al. 2001; Pelissier, Merlin et al. 2005) and associates a primary adrenal insufficiency with hypogonadotrophic hypogonadism (Wheeler, George et al. 2008). Symptoms of primary adrenal insufficiency may appear within 5 to 7 days after birth (Pelissier, Merlin et al. 2005). The disease usually evolves as a salt wasting syndrome. The newborn or infant presents with failure to thrive, feeding difficulties, including regurgitation and vomiting, diarrhea, dehydration, lethargy, hyperkalemia, metabolic acidosis and hypoglycaemia (Pelissier, Merlin et al. 2005; Limal, Bouhours-Nouet et al. 2006; Wheeler, George et al. 2008). The patient may present with an acute adrenal crisis comprising dehydration, lethargy, seizures or neurological distress, shock or sudden death, either occurring spontaneously or during stress provoking conditions like infections, gastro-intestinal disorders or surgery (Limal, Bouhours-Nouet et al. 2006). The adrenal insufficiency also results in a progressive hyperpigmentation of the skin secondary to the rise of the ACTH hormone (Pelissier, Merlin et al. 2005). Some newborns also present with persistent physiologic jaundice and hepatomegaly (Limal, Bouhours-Nouet et al. 2006; Lin, Gu et al. 2006). Without early appropriate steroid replacement, the AHC is lethal (Wheeler, George et al. 2008; Li, Liu et al. 2010). The typical adolescent presentation of AHC is a hypogonadotrophic hypogonadism (HH), characterized by puberty delay, sometimes preceded by cryptorchidism.

X-linked AHC is caused by deletions or mutations in *DAX1* gene (which stands for Dosage-sensitive sex reversal, Adrenal Hypoplasia Congenita critical region on the X chromosome, gene 1; also called *NROB1*) (Lin, Gu et al. 2006; Shaikh, Boyes et al. 2008). The *DAX1* gene consists of two exons of 1,168 and 245 bp, respectively, separated by a 3,385-kb intron, and codes for an atypical, 470-amino acid member of the orphan nuclear receptor superfamily (Zanaria, Muscatelli et al. 1994; Guo, Mason et al. 1995). *DAX1* expression has been detected in the anterior pituitary, hypothalamic ventromedial nucleus, developing adrenal cortex, adult adrenal cortex, Sertoli and Leydig cells in the testis, and theca and granulosa cells in the ovary (Zanaria, Muscatelli et al. 1994; Guo, Burris et al. 1995; Ostermann, Salvi et al. 2006). More than 100 causal mutations in the DAX1 gene have been reported so far (Phelan and McCabe 2001; Lin, Gu et al. 2006; Li, Liu et al. 2010).

To date, no data on the clinical presentation of AHC caused by *DAX1* mutations in central Africa are reported. Here we report a Congolese pedigree with many cases of unexplained male deaths and the detection of a novel *DAX1* missense mutation in an obligate female carrier.

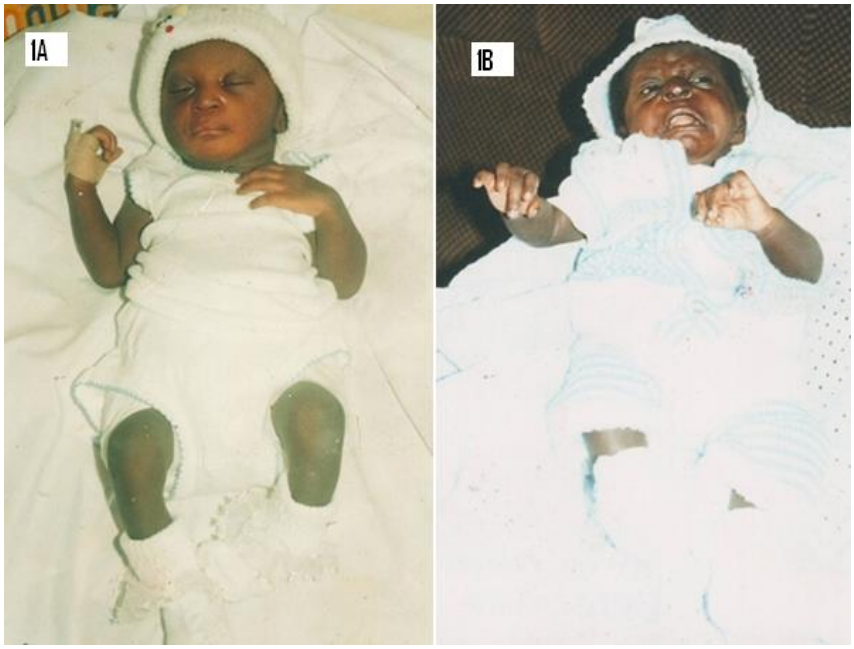
1.19.2 Case report

The proband and her husband are young, unrelated and of Congolese origin (III:10 and III:11 figure 2). She was referred for unexplained recurrent early death in her offspring. The first pregnancy ended as a miscarriage at 16 weeks during a malaria episode. The first child, a boy, was delivered at 38 weeks by C-section for maternal-fetal disproportion (figure 1A; IV:11 figure 2). After birth resuscitation was not needed. The male newborn had a birth weight of 3800 g (>P90), height of 48 cm (P25-P50), a head circumference (OFC) of 33 cm (P25-P50). No hyperpigmentation was noticed at birth neither were there dysmorphic features. At day 2, he presented with isolated jaundice. Because of a suspected neonatal infection, antibiotic treatment was installed for seven days and he received phototherapy during 4 days. Hormones and electrolytes levels were not tested. The jaundice disappeared after 5 days, and he was discharged at day 9. He presented a progressive generalized hyperpigmentation and loss of weight even though he was regularly breast fed. There were no complications after his immunizations. On day 74, he presented an acute episode with lethargy and seizures, and resuscitation at an emergency department failed. Weight at that time was 3000 g (P3=4300g). No post-mortem examination was performed. The third pregnancy evolved normally. A male infant was delivered at gestational age of 39 weeks (IV:12, figure 2). Birth weight was 3600 g (P75-P90), height 48 cm (P25-P50) and OFC 34 cm (P50-P75). He was regularly breast fed. He presented a progressive hyperpigmentation of the skin (Figure 1B). At birth, he received BCG and first oral dose of poliomyelitis vaccines without complications. At 45 days of age, he received the second dose of poliomyelitis vaccine, the first dose of DPT (Diphtheria, Pertussis, and Tetanus) and hepatitis. The immunization was followed by an episode of fever and excessive urine production. He initially received paracetamol and oral water supply at home. However, about six hours later he presented anuria, lethargy and inability to drink. He died on the way to the hospital. No autopsy was performed.

The family history revealed eleven other cases of early, unexplained deaths of male infants (Figure 2). In retrospect, it became clear that they all had presented progressive hyperpigmentation. No additional dysmorphism or malformations were noted. No biochemical tests were performed.
However, the family history, which was compatible with an X-linked condition and the clinical features of hyperpigmentation and sudden deaths, raised the possibility of X-linked Adrenal Hypoplasia Congenita. This was confirmed by the detection of a *DAX1* mutation in the proband. She carried a

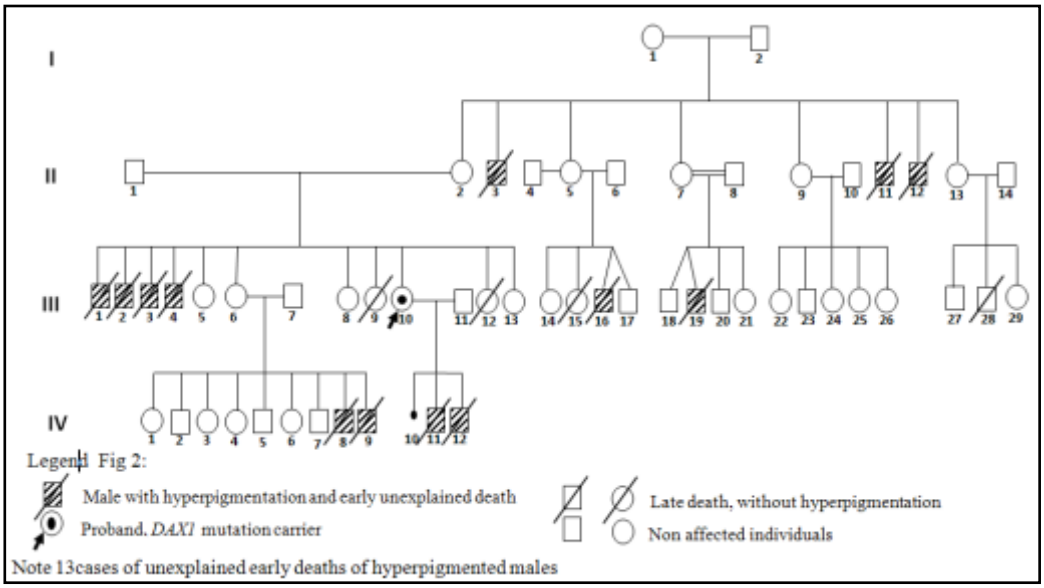
heterozygous missense mutation c.1274G>T, (p.Arg425Ile). The substitution occurs in a highly conserved domain in the second exon of gene. This substitution leads to the replacement of Arginine by Isoleucine in the putative Ligand Binding Domain (LBD) of the protein with subsequent loss of function.

Figure 1. Proband's children



Legend Fig 1: 1A: first infant on day 4. Note jaundice on the face and hyperpigmentation elsewhere. 1B: second infant on day 34. Note diffuse hyperpigmentation and failure to thrive.

Figure 2. Pedigree of the family.



Note the presence of 13 unexplained deaths in males, related through females, evoking the X-linked pattern of inheritance

1.19.3 Discussion

We present a Congolese family with genetically confirmed X-linked Adrenal Hypoplasia Congenita. The proband is a heterozygous carrier of a novel missense mutation, but two other different missense mutations in that position were previously reported: R425G and R425T (Lin, Gu et al. 2006). In retrospect, the first child in the family probably presented a progressive salt wasting syndrome, with failure to thrive, jaundice, and complicated by an acute adrenal crisis with sudden onset lethargy and seizures. The second child had normal initial evolution except for progressive skin hyperpigmentation. The triggering event was probably post-immunization fever. Two patients with acute adrenal crisis after DPT immunization were previously reported (Yang, Sujan et al. 2006).

Corticoid replacement is the key treatment for this condition, with intravenous hydrocortisone as well as saline and glucose solutions during an acute adrenal crisis (Wheeler, George et al. 2008). The outcome depends on the time of diagnosis and initiation of treatment. Altogether, 13 unexplained hyperpigmented male infants were retrospectively identified in this pedigree.

The real incidence of the CAH is not known. For the X-linked form, the incidence is estimated between 1:140,000 and 1:1,200,000 children (Lin, Gu et al. 2006). However, currently, no reports exist on the occurrence of CAH in Sub-Saharan Africa. The most likely explanation is that this condition remains undiagnosed in the majority of cases in developing countries. A number of reasons exist for this misdiagnosis, including the high prevalence of neonatal infections and the similarity in clinical presentation of adrenal insufficiency and neonatal infections, the lack of laboratories that provide routine hormone testing, the difficulty to recognize skin hyperpigmentation in a black person and the poor medical genetic knowledge. In comparison with other developing countries, the Democratic Republic of Congo has the highest Neonatal Mortality Rate. About 29 % of neonatal deaths worldwide are due to neonatal infections (Lawn, Kerber et al. 2010). Therefore, many practitioners focus their attention on infectious diseases. Also, the AHC symptoms observed in the present cases such as sudden lethargy, seizures, fever, jaundice and hypoglycaemia mimic infections or cerebral malaria in infancy. The differential diagnosis of AHC includes Congenital Adrenal Hyperplasia (CAH), Adrenoleucodystrophy and exceptionally congenital defects of hypothalamus and pituitary (Ozer, Kaya et al. 2009). To resolve this, hormone measurements are required. However, in developing countries, such testing is mostly unavailable in primary, secondary and even in some tertiary care hospitals. Another major challenge is the assessment of the skin hyperpigmentation in the black population (*Fig 1*). Clinicians have to rely on their experience in assessing skin colour, but more important is the description of the evolution of the intensity of the skin colour as reported by the parents. Finally, the

diagnosis was suggested by a careful analysis of the pedigree of this family, which leads to the recognition of an X-linked inheritance pattern. This illustrates the necessity for medical and clinical genetics to be part of the curriculum of medical school in developing countries.

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1.20 A novel heterozygous mutation of three consecutive nucleotides causing Apert Syndrome in a Congolese family

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Running title: Apert syndrome in Congolese family

1.20.1 Introduction

Apert syndrome (OMIM 101200) is a rare genetic condition with an estimated prevalence of ~1 in 70,000 live births and presenting craniosynostosis (premature and antenatal fusion of skull sutures) associated with syndactyly of hands and feet (Ibrahimi, Eliseenkova et al. 2001; Bochukova, Roscioli et al. 2009). The phenotype can be variable, both regarding to the severity of the acrocephalosyndactyly or to the presence of additional manifestations (such as cleft palate or intellectual disability) (Park, Theda et al. 1995; Oldridge, Lunt et al. 1997; Lajeunie, Cameron et al. 1999; Mundhofir, Sistermans et al. 2013). However, this variability cannot readily be explained by the underlying causal mutations. Indeed, majority of patients present with single nucleotide substitution in the linker region between the immunoglobulin-like domains II and IIIa (IgII and IgIIIa) of the ectodomain of the Fibroblast Growth Factor Receptor 2 (*FGFR2*, OMIM 176943) (Wilkie, Slaney et al. 1995; Oldridge, Lunt et al. 1997; Lajeunie, Cameron et al. 1999; Sakai, Tokunaga et al. 2001). Consistent with other paternal age effect mutations, those causing AS exclusively originate during spermatogenesis, have a gain-of-function (GOF) effect and exhibit a remarkable enrichment in spermatogonia with ageing due to a protein-driven selective advantage from the mutant protein (Risch, Reich et al. 1987; Ibrahimi, Eliseenkova et al. 2001; Goriely, McVean et al. 2005; Bochukova, Roscioli et al. 2009; Goriely and Wilkie 2012). As expected for any GOF mutation, the repertoire of mutations causing AS is limited. Two single nucleotide substitutions in the *FGFR2* gene, c.755C>G; p.Ser252Trp (65 %) and c.758C>G; p.Pro253Arg (34 %), are recurrent in Apert Syndrome (Lajeunie, Cameron et al. 1999).

Besides the two canonical single nucleotide mutations, rare consecutive double nucleotide substitutions involving the CpG dinucleotide at position 755_756 and changing Ser252 into Phenylalanine (c.755_756delCGinsTT; p.Ser252Phe and c.755_756delCGinsTC; p.Ser252Phe) have been reported in Apert syndrome (Oldridge, Lunt et al. 1997; Lajeunie, Cameron et al. 1999; Goriely, McVean et al. 2005). The rarity of p.Ser252Phe mutation is ascribed to the requirement that two consecutive nucleotides of the Serine at codon 252 need to be mutated (Goriely, McVean et al. 2005).

Oldridge et al. reported the only patient carrying consecutive triple nucleotide substitutions in the linker region (c.755_757delCGCinsTCT) resulting in the change in two consecutive amino acids (Ser252Phe and Pro253Ser). Although Ser252Phe substitution is known to cause Apert syndrome, the patient carrying both Ser252Phe and Pro253Ser substitutions presented with the less severe Pfeiffer syndrome (Oldridge, Lunt et al. 1997; Kan, Elanko et al. 2002).

Here we report a male patient and his mother, both affected with Apert syndrome, carrying a new heterozygous mutation and showing variable expression.

1.20.2 Clinical report

1.20.2.1 Case descriptions

A 42-day-old Congolese male infant was referred to genetic consultation at the University Hospitals, University of Kinshasa because of congenital malformations. He was the only child of unrelated parents and was born after an uneventful pregnancy and delivery. At birth, he presented with craniofacial and limb anomalies (Figure 1). At the time of consultation, his weight was 7.7 kg (P90), length 68 cm (P97=67.5 cm) and OFC 41 cm (P75-90). Craniofacial anomalies included craniosynostosis, midfacial retrusion, mild proptosis, hypertelorism, strabismus and tented upper lip (Figure 1A&B). The ears were mildly low set with overfolded helices. There was a bilateral symmetric type 2 hand syndactyly, according to the classification by Cohen and Kreiborg (Cohen and Kreiborg 1995), and bilateral post-axial polydactyly. His feet showed bilateral and symmetrical type 1feet syndactyly (Cohen and Kreiborg 1995). The palate was normal.

The 44-year-old father, unavailable for clinical examination, was reported to have normal development and to be free of obvious malformations. The index's mother was born from a 49 years old father and was 33 years old when she gave birth to the index. She had mild intellectual disability, OFC of 53 cm (P10), length of 176 cm (P90-P97), no major craniofacial malformations but exhibited mild exophthalmos, externally rotated ears with unfolded helix (Figure 1H&I). There was symmetrical and bilateral type 1 hand syndactyly, bilateral medial deviation of toes, more pronounced on the first toe, and type 1 feet syndactyly of (Cohen and Kreiborg 1995).

The maternal grandfather had died by the time of consultation as well as 3 maternal uncles of the index. None of them reportedly had facial or limb malformations.

Figure 1. Photographs of patients

Legend: **A-G**: index. **A** & **B**: Frontal and lateral view of the index: note prominent front, depressed midface, exophthalmos, hypertelorism, strabismus, overfolded helix, anteriorly rotated lobule and low set ears, tented upper lip, oxycephalic skull ; **C, D** & **E**: Index's hands. Note abnormal position of the thumb and syndactyly of fingers II-IV; **F**&**G**: index, bilateral syndactyly of toes H-L: index's mother. **H**&**I**: frontal and lateral view of the mother's head. No major skull malformations observed. Note exophthalmos, protruding ears with unfolded helix; **J**: bilateral syndactyly of fingers II-V; **K**&**L**: toe syndactyly on right and left foot.

Radiographic investigations (Figure 2) confirmed syndactyly in the mother (type 1) and in the index (type 2 in hands and type 1 in feet), showed deviation of thumbs and duplication of terminal fifth phalange, classical type 1first metatarsal pattern, described by Cohen et al. (Cohen and Kreiborg 1995) as deviated first metatarsal of halluces with proximal phalanx located on its fibular side, in the index; bilateral carpal-metacarpal coalition made up with proximal fusion of fourth-fifth metacarpal and fusion of carpal bones made, and small extra bone between first and second metacarpal of thumbs in his mother.

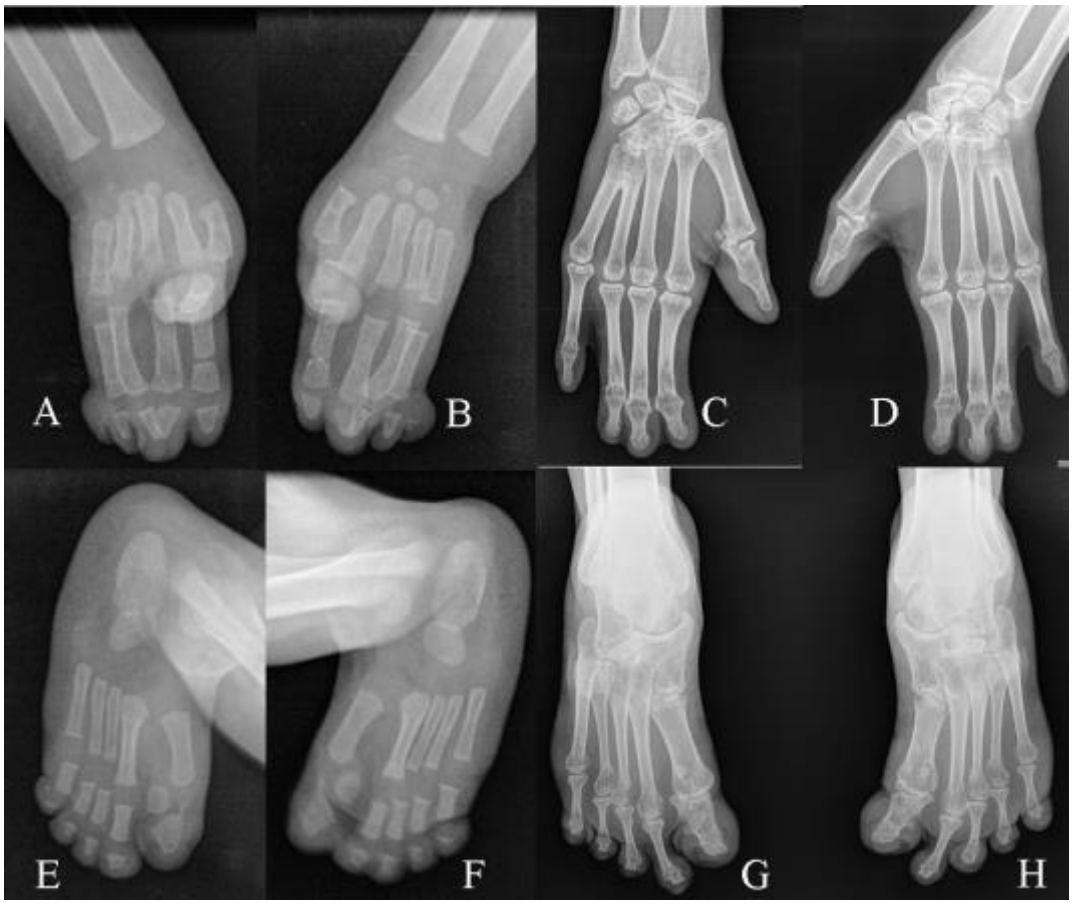

Figure 2. Radiographs of hands and feet

Legend: index's hands showing deviation of thumbs and duplication of terminal fifth phalange (A&B); Mother's hands presenting with bilateral carpal-metacarpal coalition made up by proximal syndactyly of fourth-fifth metacarpal and fusion of carpal bones, and small extra bone between first and second phalanx of thumbs (C&D); Index's feet with classical type 1 first metatarsal pattern (E&F); mother's feet with fibular deviation of first metatarsal of great toe and coalition tarsal bones (G&H).

1.20.2.2 Molecular testing

Genomic DNA were extracted locally in the Genetics laboratory, Kinshasa from venous blood by the salt saturation method as previously described (Miller, Dykes et al. 1988). The exon seven of the Isoform 1 of the *FGFR2* (RefSeq NM_000141.4) was amplified using the following M13-tagged primer pairs:

Forward primer 5'-TGTAAAACGACGGCCAGTGAGGTCACTGACAGCCCTCT-3' and Reverse primer 5'-CAGGAAACAGCTATGACCCCCCTACTCCATAGTTCCCTTC-3'.

The 50 µl amplification reaction contained 5 µl of DNA (50 ng), 5 µl of PCR reaction buffer+Mg^{2+} (Roche), 5 µl of each Primer solution (2.5 pmol/µl), 5 µl of dNTP's (2 mM), 0.5 µl (2.5 U) of Taq DNA Polymerase (Roche) and 24.5 µl of Ultrapure water (Baxter). The PCR was run on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA 94404 USA) using the following program: initial denaturation stage

at 95 °C for 5 minutes; 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 45 s; and a final extension stage at 72 °C for 5 minutes. PCR products were sequenced on ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA 94404 USA) using Big Dye termination method.

Both the mother and her son carry a substitution of three consecutive nucleotides in the linker region: RefSeq NM_000141.4:c.756_758delGCCins**CTT** (NC_000010.10:g.123279674_123279676delinsAAG). The mutation overlaps two adjacent amino acids and results in a synonymous mutation for Serine at position 252 and in a missense mutation for neighbouring Proline (p. Pro253Phe) (Figure 3). There was no evidence for mosaicism in the mother.

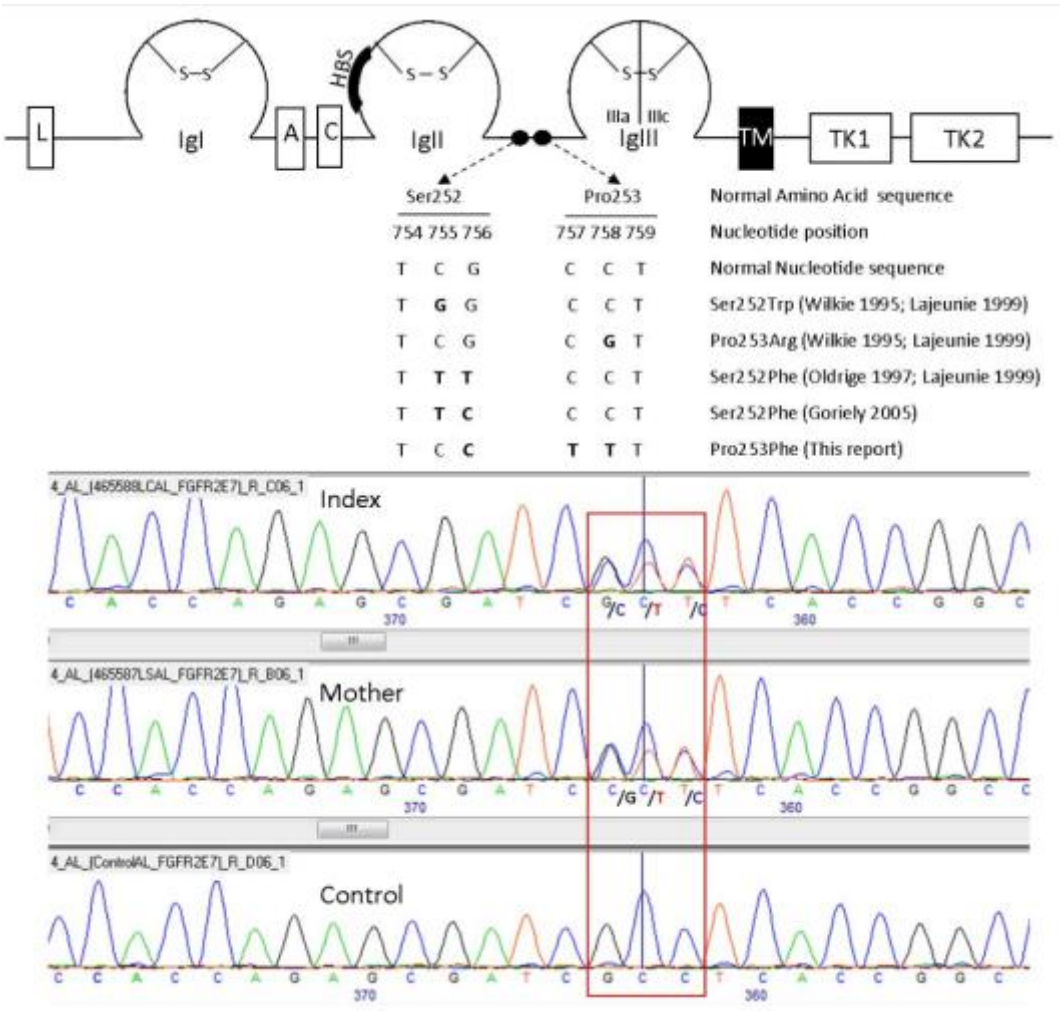

Figure 3. Structure of FGFR2, previous and current mutations

Legend: The top panel shows the FGFR2 structure. Amino acid Ser252 and Pro253 are marked by filed black ovals. L, Leader sequence; IgI, IgII and IgIII for Immunoglobulin-like domains 1, 2 and 3 respectively; A, Acidic box; C; CAM homology domain; BHS, heparin-binding site in the IgII; TM, Transmembrane helix; TK1 and TK2 represents spit-Tyrosine Kinase domain. Middle panel shows the normal amino acids sequence at positions 252 and 253 in the IgII-IgIIIa linker region, their nucleotide counterpart, and previous and current mutations in the region responsible for Apert Syndrome. The bottom panel consists of electropherograms showing mutation in the index and his mother for the current report. Nucleotides affected by the mutation are surrounded by the red box.

We analysed 210 chromosomes from 105 unrelated Congolese screening for population specific polymorphisms in the linker region by sequencing (data not shown). None of them had variations in this region.

This new mutation was submitted to ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/intro/) and received the accession number (rs387907372: delGCCins**CTT**).

1.20.3 Discussion

We present a Congolese infant with the classical phenotype of Apert syndrome, i.e. severe craniosynostosis and syndactyly of hands and feet. His mother was less severely affected, presenting mainly limb malformations. Such an intrafamilial variability was previously reported in monozygotic twins (Breugem, Fitzpatrick et al. 2008) and remains unexplained.

FGFR2 mutations causing Apert syndrome mostly occur during spermatogenesis and exhibit a very strong paternal age effect (Goriely, McVean et al. 2003; Goriely and Wilkie 2012). The fact that the maternal grand-father was 49 years at the moment the index's mother was born is likely a risk factor in this family.

Interestingly, we observed a novel, previously undescribed *FGFR2* mutation causing Apert syndrome: **p.Pro253Phe**. Consistent with the previously reported AS causing mutations, this new mutation substitutes the Proline by a more bulky side-chain amino acid (Grantham 1974; Dufton 1997; Bochukova, Roscioli et al. 2009). This is the second alternative amino acid mutation ever reported affecting the proline at position 253 after the p.Pro253Arg (Lajeunie, Cameron et al. 1999).

Genotype-phenotype correlations in Apert syndrome suggested that mutations of residue Ser252 were correlated with more severe craniofacial defects whereas mutations of residue p.Pro253 were associated with more severe limb anomalies (Lajeunie, Cameron et al. 1999; Ibrahimi, Eliseenkova et al. 2001; Agochukwu, Solomon et al. 2013). The absence of obvious skull defect in the mother together with carpal coalition would be consistent with this observation.

A second intriguing observation is the presence of a mutation in three consecutive nucleotides: c.756_758delGCCinsCTT. Such variants, multiple nucleotide substitutions are not frequently observed in the human genome (Smith, Webster et al. 2003) and are rare in human genetic diseases (Kondrashov 2003). Three Apert syndrome patients have been reported carrying consecutive double nucleotide substitutions in the linker region (c.755_756delCGinsTC and c.755_756delCGinsTT) leading to p.Ser252Phe substitution and to Apert syndrome (Oldridge, Lunt et al. 1997; Lajeunie, Cameron et al. 1999; Goriely, McVean et al. 2005). Oldridge et al. reported one patient with a triple consecutive nucleotide substitutions in the linker region (c.755_757delCGCinsTCT) changing Ser252 and

Pro253into Phe and to Ser respectively. Despite the presence of Phe at position 252, the mutation resulted in Pfeiffer syndrome, a less severe form of acrosyndactyly (Oldridge, Lunt et al. 1997). Substitution of multiple nucleotides may in fact reflect *trans*-located single nucleotide mutations. Maternal family members were not available for testing, but transmission of all 3 nucleotide variants from mother to son together with absence of polymorphism essentially proves that all three are in *cis*.

The mechanism leading to the substitution of multiple adjacent nucleotides is still under discussion. First, the substitution of multiple consecutive nucleotides also termed Tandem Base Substitution (TBS) may have occurred in one single event (Averof, Rokas et al. 2000; Kondrashov 2003; Bazykin, Kondrashov et al. 2004; Chen, Ferec et al. 2013). Depending on the size of the TBS, mechanisms may include transient hypermutability, chromothripsis, gene conversion, in situ inversion or Alu insertions (Whelan and Goldman 2004; Bochukova, Roscioli et al. 2009; Chen, Ferec et al. 2013). Second, the mutations may have arisen as a number of independent consecutive events, each affecting one nucleotide at once (Averof, Rokas et al. 2000; Whelan and Goldman 2004). Such a pattern was previously observed in a patient with *FGFR2*, 755_756CG>TC substitutions whose mother was normal and father was heterozygous carrier of a 755C>T substitution only. (Goriely, McVean et al. 2005). When applied to our case, three putative first events could be proposed: c.756G>C (p.Ser252Ser, a synonymous mutation), c.757C>T (p.Pro253Ser) or c.758C>T (p.Pro253Leu). Of the three possibilities, the first appears to be the most likely first event, since it may remain silent and allow adjunction of further more pathogenic hits as in the report from Goriely et al. 2005. This unique mutation is the first mutation ever described in an Apert patient from Central Africa. Therefore, we hypothesized that our patients could harbour two true mutations in association to an adjacent population specific variant, c.756G>C synonymous SNP. In order to verify this assumption, we reviewed existing databases. The c.756G>C was not reported in any of them; however, a c.756G>A (C>T on the positive strand) synonymous variant was reported in one African-American chromosome out of 13005 total chromosomes in the ESP cohort population (**rs138769323** in *db*SNP builds 134-137), allowing us to calculate a prevalence of 0.00015. Although this region was not known as polymorphic in available databases, and given that sequence data from Congolese population are not available in existing databases, we undertook to exclude a putative Congolese specific polymorphism. We sequenced the *FGFR2* exon IIIa in 105 controls DNA samples (210 chromosomes) from the Congolese population. These samples came from clinically normal parents of patients with Intellectual Disability/Developmental Delay (ID/DD) recruited in Kinshasa, DR Congo in the framework of a study on the genetics of ID/DD. None of the controls carried a nucleotide variation at nucleotide positions 756_758.

In conclusion, we report on a novel and unique mutation involving three adjacent nucleotides. The present report underscores the usefulness of reaching a molecular diagnosis, even in cases with a well-known genetic condition and where the spectrum of mutations is known. Only by this way rare and novel mutations can be discovered, leading to further insight into their pathogenesis. Moreover, it is tempting to speculate that the present observation is linked to the patients' Central African origin, a population which is poorly accessible for genetic studies thus far.

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The authors declare no conflict of interest.

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The main aim of this study was to contribute to the understanding of the genetic aetiology of ID in Central Africa. For the first time, we present data on a comprehensive and integrated clinical and (molecular) genetic study on the aetiology of ID in a central African country, the DR Congo.

1.21 Understanding the genetic aetiology of ID in Kinshasa

Overall, in the cohort we investigated, we find likely genetic causes in 34/127 patients (26.8 %). This yield is very similar to studies performed in other countries (Battaglia, Bianchini et al. 1999; Rauch, Hoyer et al. 2006; Bernardini, Alesi et al. 2010). Likewise, as in other studies before the introduction of NGS, chromosomal aberrations are the most common cause of ID, with Down syndrome being the most frequent (Battaglia, Bianchini et al. 1999; Devriendt, Holvoet et al. 2003; Rauch, Hoyer et al. 2006; Ropers 2008). In 10 out of 86 (11.62 %) patients with ID of clinically unknown aetiology we have found a submicroscopic chromosomal imbalance, and of interest, 4 of the 11 CNV's were recurrent CNV's, caused by a flanking low copy repeats. We did not identify a single fragile X patients. However, in this study we have confirmed an X-linked recessive disorder in two brothers, i.e. Partington syndrome and in one boy, a possible X-linked disorder caused by a variant in the TAF1-gene was identified. By means of exome analysis, we identified 1 possible autosomal recessive metabolic disorder. The majority of cases (65 %) remains without diagnosis, which compares well to other studies in the Caucasian population, where, using a similar approach, a diagnosis is rarely reached in more than 50% of patients (Battaglia, Bianchini et al. 1999; Devriendt, Holvoet et al. 2003; Rauch, Hoyer et al. 2006).

The recent introduction of exome studies, which allow an unbiased genome-wide screening for SNV's causing ID, has shown that the aetiology if ID is highly diverse. Nevertheless, mutations in certain genes are more prevalent. For instance, mutations in *ARID1B, SATB2, SYNGAP1, ANKRD11, SCN1A, DYRK1A, STXBP1, MED13L* were observed in 0.5 to 1% of cases (Deciphering Developmental Disorders Study. 2015) (DDD 2015). It will be of interest to study a larger cohort of "unknown" Congolese cases with ID by means of exome analysis, to confirm our hypothesis that, similar to the CNV's, the same monogenic disorders are also frequent in Central Africa.

The results of this study show for the first time that genetic factors are a major cause of ID in the DR Congo, as observed in other countries. Moreover, the same disorders were diagnosed as elsewhere. For the majority of the conditions diagnosed, such as Partington and Smith-Magenis syndrome and the various chromosomal imbalances, these represent the first cases ever reported in this part of the

world. Whereas the scientific value of reporting “the first case” of a specific syndrome appears to be limited from a Western point of view, this has significant impact on the local community. This is important evidence to fight false believes concerning the cause of ID and congenital malformations. To date, most persons still hold the opinion that ID has a supernatural cause (Abasiubong, Obembe et al. 2008; Kromberg, Zwane et al. 2008; Njenga 2008). In teaching health care workers and educating the public at large on ID, the fact that genetics have an important role can now be illustrated by data from our own country.

1.22 Limitations of the study

The diagnostic process was limited due to local circumstances.

First, no imaging studies could be performed. For instance, in children with developmental delay or ID with macrocephaly or microcephaly, brain imaging is standard practice (Battaglia, Bianchini et al. 1999; Balci, Sawyer et al. 2015). Obviously, more diagnoses could have been made if this was available. Likewise, the suspected diagnoses of acquired brain injury could have been substantiated.

Also, the genetic studies were not complete in all patients. One obvious reason is that exome analysis is still very expensive and could therefore only be performed in a subset of well-selected cases. Likewise, for financial reasons, microarray-CGH was done using recycled slides. Our protocol to strip the slides did perform well for 60k slides but not for 180k slides, resulting in a less than optimal resolution for our microarray-CGH analyses. Also, some DNA samples were missing. One reason is that access to parents was difficult. Parents were met during the recruitment process and sometimes a second time when results were communicated during one of the follow-up visits to Kinshasa. Often, only one of the parents was available, mainly the mother. Also, since genetic analyses were done in Leuven, biological samples had to be transported from Kinshasa to Leuven. The quality of certain samples suffered from this, and as a result, we did not obtain genetic data on the complete cohort.

The present results are not necessarily representative of all children with ID in Kinshasa. There is a clear bias in the recruitment of the patients, who were ascertained in institutions and schools for children with ID. In the absence of a public schooling or social security system, parents face a high cost to send their children to school. Therefore, the families we encountered during our study are mostly privileged ones. Also, very few children presented cerebral palsy, despite the fact that from other sources we know that this is common in developing countries (Durkin 2002; Bergen 2008; Van Rie, Mupuala et al. 2008; Adnams 2010). From our contacts with a parents’ association for children with ID, we learned that the majority of them suffered from cerebral palsy. Likely, these children often do

not attend the schools we included in our study. Also, only very few children present with a major malformation. It is likely that this is associated with a high mortality, but also a reduced likelihood of receiving school education.

We did not diagnose a single case of Fragile-X syndrome in our cohort. It is unlikely that Fragile-X syndrome is absent in this country. Most likely, this is due to the small size of the study (including only 94 boys), but may also be explained by a combination of factors causing a recruitment bias. The distribution of *FMR1* CGG alleles is similar to other African groups and African-American (Crawford, Zhang et al. 2000; Peprah, Allen et al. 2010; Essop and Krause 2013). We have identified 2 carriers of intermediate alleles, which is an indirect clue that fragile X patients could be identified in a bigger cohort. In order to get more insights in the incidence and presentation of Fragile-X syndrome, we plan to focus on a cohort of familial ID, i.e. families with affected brothers or with pedigrees compatible with an X-linked inheritance. Also, screening of a cohort of new-borns to determine the incidence of the *FMR1* premutation will yield more information.

1.23 Clinical parameters and the chance of reaching an etiological diagnosis

We also wished to define clinical parameters associated with a higher chance of reaching an etiological diagnosis. Especially in a country where access to expensive genetic testing is almost absent, a clinical approach will remain the cornerstone of reaching an etiological diagnosis. In this study, we have reached a clinical diagnostic in 24 out of the 127 patients (19 %). The clinical diagnosis of syndromes is challenging and requires an expertise that can only be gained by training under supervision by experienced experts. We therefore explored how certain clinical data could predict the likelihood of reaching a specific diagnosis or aid in the selection for further genetic testing.

Previously, it has been shown that the presence of dysmorphism and/or major malformations increases the likelihood of detecting a chromosomal imbalance (Breckpot, Thienpont et al. 2010; Battaglia, Doccini et al. 2013; Shoukier, Klein et al. 2013). Our study is consistent with this, though the number of cases with a major malformation or with a chromosomal imbalance was too small to allow a proper statistical evaluation. Eight out the 10 individuals with a chromosomal imbalances found by microarray-CGH were dysmorphic, with the exception of the patient with a del15q11.2 and the carrier of the del2q24.3.

Another way to reach a syndrome diagnosis is to replace the “human eye” by an expert syndrome recognition system starting from digitalized facial pictures. Syndrome databases such as POSSUM or London Dysmorphology Database allow the selection of syndromes matching the description of

features in a case. However, limiting factors include variability in expression of most syndromes, the difficulty to accurately and objectively describe dysmorphic features and often, the lack of specific features. Computer analysis of digitalized 2D or 3D pictures has been applied as a research tool, and more recently, user-friendly tools have been made available to support the clinical diagnosis of children with unknown syndromes. The most advanced of these systems if Face2Gene (http://www.fdna.com/face2gene/). The potential of such a system is huge, especially in an environment where access to expertise is lacking. Imagine the situation of a child born in a small village in Central DR Congo, with a syndromic aspect and a suspected diagnosis of Down syndrome or trisomy 18. Uploading a picture of the child onto the online tool may support or refute this diagnosis. The logistics needed for this are accessible to any physician, since mobile telephones are an established means of communication in the country. However, performance depends on a high specificity and sensitivity of the tool. In its turn, this depends on training the system by a large number of clinical pictures of existing patients. Therefore, on can expect that such systems will probably only perform ideal for the more common syndromes. In addition, and more importantly for developing countries, we have shown that training with Caucasian patients only is not sufficient (see also discussion below).

Testing for fragile X is one of the routines testing for ID in Western countries. In limited resource setting, it is very important to preselect patients for further testing and avoid unnecessary and costly testing. For that reason, pre-screening tools such as clinical checklists may be invaluable in Africa. Many studies have designed, used or adapted checklists to screen potential fragile-X patients (Hagerman, Amiri et al. 1991; Giangreco, Steele et al. 1996; de Vries, Mohkamsing et al. 1999; Maes, Fryns et al. 2000; Guruju, Lavanya et al. 2009). In this study, we explored the usefulness of 3 fragile-X screening lists. Since fragile-X is absent in our cohort, we cannot draw final conclusions about the checklists. However, one remarkable result with these checklists is the large number of false positives, mainly due to the high prevalence of behavioural characteristics such as hyperactivity. This may indicate that existing screening lists for other conditions will need to be adapted to the local situation. When Congolese fragile X patients will be identified, it will be interesting to pursue the assessment and improvement of the checklist for their use in the DR Congo.

1.24 Variability in expression of genetic syndromes

Another aim of this study was to gain insight into the variability in clinical expression of specific genetic disorders in Central Africa.

We diagnosed several conditions clinically, e.g. Down syndrome, Partington syndrome, Williams syndrome. Clearly, the features in these conditions are very similar to those observed in the West. However, there are differences in facial features.

Differences in clinical presentation may result from differences in ethnic background. As previously stated, Face2Gene is a tool that compares in an objective way the digitalized facial features of a child with dysmorphic features with a library of known syndromes. When we analysed a cohort of Congolese individuals with Down syndrome we noted that they were less easily recognized as Down syndrome compared to Flemish Down syndrome cases. This shows that there are ethnic differences in the facial presentation of Down syndrome, corroborating previous reports that the facial phenotype has ethnic differences for certain syndromes (McDonald-McGinn, Minugh-Purvis et al. 2005; Talbert, Kau et al. 2014; Tekendo-Ngongang, Dahoun et al. 2014).

Also, there appears to be differences in behavioural aspects. From the Fragile-X screening lists, we noted that many children have high scores on behavioural items such as hyperactivity. Whether this is a true difference in behaviour or is due to over-scoring by the parents remains to be investigated. Of interest, we did not clinically recognize the child with Smith-Magenis syndrome. One reason is that the behavioural aspects, which typically are an important guide towards the diagnosis, did not stand out in this child. No sleeping difficulties were reported either. More research is needed into possible differences of behavioural phenotype for this and other syndromes.

The variability in clinical expression may result from effects of environmental factors on the course of the disease. One of the main differences with studies in developed countries is the environment in which Congolese children grow up. Birth injury, infectious diseases, malnutrition, etc, have a major impact on the health of children (Durkin 2002; Bergen 2008; Van Rie, Mupuala et al. 2008; Adnams 2010). Indeed, we also found that in our cohort of patients, infections (such as meningitis) were common. However, in only few of them, an acquired brain insult was thought to be the cause of their ID. On the other hand, infections may influence the clinical manifestations, and one risks to overlook a genetic diagnosis, masked by an intervening environmental insult. For instance, we describe a young boy with the classical somatic phenotype of Williams syndrome. Surprisingly, he has severe ID and absent speech. The genetic mutation was the recurrent 1.57 Mb deletion in 7q11.23 causing this

syndrome. However, at age 2 years, he suffered from meningitis and coma, and lowered intellectual capacities, most likely due to brain damage. The same is also illustrated by the family we diagnosed with congenital adrenal hypoplasia, an X-linked condition. The family presented with multiple boys dying at young age due to infections. A suspicion of a genetic cause was raised when the pedigree revealed an X-linked inheritance pattern. Additional signs were also present, including the darker skin pigmentation (melanodermia), due to an increased ACTH production.

Last but not least, the difference may just be due to the subjective interpretation by the clinicians. It is well known that the description of the phenotype is a subjective process that is influenced by the level of training and the experience of the clinician. In this study, we have shown that the ethnic origin of the clinician also influences his perception of clinical phenotype. Only medium inter-ratter agreement was found between 5 African clinicians and 5 Europeans when we ask them to score the facial gestalt of 127 Congolese patients.

1.25 Causes of and variability in genetic mutations observed in Congolese patients with a genetic disorder

During our doctoral training, we came across with a Congolese family with a child and his mother presenting typical features of Apert syndrome. About 99 % of cases are explained by 2 amino acid changes. Interestingly, the mutation analysis revealed a new mutation involving 3 consecutives nucleotides in the hotspot for Apert syndrome. This new mutation expanded the Apert syndrome mutation spectrum. However, it is remarkable that the first patient with Apert syndrome ever to be studied genetically in Central Africa carries a highly unusual mutation, raising the possibility that the specific African genetic background could somehow be at the basis of this.

Currently, very little is known about the causes of mutations. Advanced maternal age is the main risk factor for aneuploidies. The advanced maternal age in the majority of Down syndrome cases in our cohort is in agreement with this. Advanced paternal age is a risk factor for de novo SNV's. However, our study cannot address this question. One of the well-known mechanisms of copy number variations are unequal crossing-overs between low copy repeats (LCR). This results in recurrent, relatively common microdeletions or duplications, which share a same size (flanked by the LCR's) and often a recognisable phenotype. Also in our study, of the 11 submicroscopic imbalances, 4 were recurrent ones. This indicates, not surprisingly, that the same mechanism also operates in the Central-African genome.

1.26 Future prospects

This study, its results and limitations highlight challenges for the introduction of human and medical genetics in developing countries and illustrates also the opportunities for such an exciting discipline in Africa. The main challenge now is to translate these findings to provide a better care for patients and families.

From a clinical point of view, it is clear that a genetic evaluation must be part of the workflow of any child with ID. However, this requires knowledge and expertise in this field. The work performed during this thesis has significantly contributed to this. Next, once an etiological diagnosis is reached, the existing guidelines for the follow-up and guidance of individuals with specific syndromes should be implemented. Given the variable environment, the existing protocols should be evaluated and probably be adapted. This will be mostly important for the more frequent syndromes. A future study we therefore envisage is a clinical study in children with Down syndrome, evaluating the different medical and developmental manifestations and their treatment, based on the existing international guidelines.

Finally, the data should be communicated to the families in the genetic counselling process. This is challenging, since we must take into account the specificities of each population. A person's needs are influenced by social, cultural, religious and legal factors that vary in different regions around the world. Future research in these aspects in the local population is therefore needed, in order to implement successfully genetic diagnosis for ID in the DR Congo.

Following the clinical evaluation, genetic testing is a second cornerstone of the diagnostic process. Karyotyping is being introduced in the laboratory of the genetics centre in Kinshasa, since it is essential in the diagnosis of recurrent miscarriages, infertility, and useful for certain clinically recognizable aneuploidies such as trisomy 13, 18 and 21 as well as Turner syndrome. Also, for Down syndrome, karyotyping is essential to exclude a Robertsonian translocation. However, for genetic testing in individuals with unexplained ID, chromosomal microarray is currently the method of choice, since it allows the detection of the frequently occurring smaller chromosomal imbalances, below the resolution of classical karyotyping. In our study, only 3 of the 10 cases diagnosed by microarray-CGH would have been detected by standard karyotyping: a 11.9 Mb deletion on chromosome 8p, 50 % mosaic deletion of 47.9 Mb on chromosome 13 and a 33 Mb chromosome 20 duplication.

However, in the current situation, the introduction of microarray-technology in the laboratory in Kinshasa is problematic. There is the high investment cost for the equipment and difficulties in

obtaining specialized support for its installation and maintenance, and there is the absence of locally available consumables. Therefore, one needs to consider alternatives. Multiplex Ligation-Dependant Probe Amplification (MLPA) allows the detection of several tens of different targeted chromosomal imbalances in one single reaction, at a reasonable cost. Equipment for the quantitative analysis of the fragments is less sophisticated than that required for microarray-CGH. Commercial kits are available that target the most common microdeletion syndromes or the subtelomeric regions. In the present study, 3 of the 10 imbalances detected by microarray-CGH could be identified by the SALSA MLPA P245 Microdeletion Syndromes-1 probemix: 2p16 deletion, Smith-Magenis syndrome, 22q13 deletion. Combining standard karyotyping and this MLPA kit, 6/10 cases would have been diagnosed. This figure is similar to the study of Jehee et al. (2011) (Jehee, Takamori et al. 2011): the combination of MLPA and karyotyping detected 76.45 % of imbalances that were found by karyotyping and chromosomal microarray. An approach whereby a first screening is done by means of karyotyping and/or MLPA therefore offers an attractive alternative in a resource poor setting.

On the long term, it is likely that whole genome sequencing, once its price will drop will become the standard tool to screen the entire genome for all types of mutations (SNV's, CNV's, balanced structural anomalies). This will become the first tier test, both in diagnostics and research. Likely, DNA sequencing could be done in one centralized (commercial) unit followed by data analysis locally, incorporating the clinical data. This might, at least in part, solve the problem of the lack of well-equipped molecular genetic laboratories in the country. However, the tools for bio-informatics analysis need to be established. Currently, the lack of fast internet access and frequent cuts in electricity supply make this problematic.

Nevertheless, we believe that the anticipated introduction of whole genome sequencing as a cheap research tool should guide us in our future research. One of the opportunities of genetic studies in Central Africa is the possibility to identify novel genes or mutations for conditions which are unique to this region. One should therefore establish clinically well-defined cohorts of patients with rare diseases and store DNA from cases and their parents. In the field of common, multifactorial diseases, the unique environment in Central Africa will offer the possibility to gain insight in complex gene-environment interactions.

At the onset of this study, the only contribution in the field of genetics were the courses in medical genetics given by Prof. P. Lukusa at UNIKIN and casual teaching visits by the team from the Centre for Human Genetics in Leuven, Belgium. Now, 5 years later, the situation has changed completely. The Congolese Society for Human Genetics has been installed, offering a forum for the emerging human

and medical genetics society in the country. A genetics centre was installed in Kinshasa, offering regular genetic clinics and teaching in medical genetics. A genetics laboratory has been installed at the faculty of medicine at the University of Kinshasa and INRB, which supports genetics diagnosis and research, mainly through a DNA extraction facility. These circumstances offer a more secure basis for future research into the genetics of ID. These are first but important steps toward providing patients with ID and their families the same level of care as in the rest of the world.

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PROFESSIONAL PROFILE

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Tite Minga Mikobi, **Aimé Lumaka**, … Gert Matthijs, Valerie Race. (2015). Copy Number polymorphisms of Alpha-Globin genes in Congolese Sickle Cell Anemia Patients and their hematological influence. (***Submitted to Plos One***)

Aimé LUMAKA, Rita LUKOO, Gerrye MUBUNGU, Paul LUMBALA, Gloire MBAYABO, Aimée MUPUALA, Tshilobo Prosper LUKUSA, Koenraad DEVRIENDT. WILLIAMS-BEUREN Syndrome: Pitfalls for diagnosis in limited resources setting. (***Submitted to Clinical Case Report***)

Michel Ntetani Aloni; Tite Minga Mikobi; **Aimé Zola Lumaka**; Didine Kinkodi Kaba; Jean Marie Mbuyi-Muamba; Prosper Lukusa Tshilobo; Koenraad Devriendt; Gert Matthijs; Valérie Race. (2015). Protective *BCL11A* and *HBS1L-MYB* polymorphisms in Congolese Sickle Cell Anemia Patients. (**Submitted to Plos One**)

Toni Kasole LUBALA, **Aimé LUMAKA**, Sébastien MBUYI-MUSANZAYI, Olivier MUKUKU, Didier MALAMBA-LEZ, Oscar Numbi LUBOYA, Prosper LUKUSA-TSHILOBO. (2015). Evidence of fragile X syndrome with mosaic size mutation in Bantu patient from Central Africa. (**Submitted to Pediatrics**)

Participation at international meetings

Meeting	Venue	Date	Title presentation	Oral/ poster
Joint EuroGentest/Techgene Scientific meeting	Leuven	10/02/2011	n/a	n/a
11th Congress of the Belgian Society for Human Genetics	Louvain-La-Neuve	04/03/2011	n/a	n/a
22nd European Dysmorphology Meeting	Le Bischenberg, Alsace-France	31/08/-02/09/2011	Variability in expression of chr14q22.1-22.2 microdeletion	Oral
12th Congress of Belgian Society of Human Genetics	Liège Palais des Congres	02/03/2012	An etiological diagnostic survey in 46 males	Poster
23rd European Dysmorphology Meeting	Le Bischenberg, Alsace-France	5-7/09/2012	A genetic etiological study of intellectual disability in the Democratic Republic of Congo. Initial results	Oral
13th Congress of the Belgian Society for Human Genetics	Brussels Expo, Belgium	15/03/2013	A novel heterozygous mutation of three consecutive nucleotides causing Apert syndrome in a Congolese family	Poster
1st Congress of the Congolese Society for Human Genetics	Sultani Hotel, Kinshasa	27-29/05/2013	Genetic causes of intellectual disability among males in Kinshasa	Oral
24th European Dysmorphology Meeting	Bischenberg, Alsace-France	6/09/2013	A novel heterozygous mutation of three consecutive nucleotides causing Apert syndrome in a Congolese family	Oral

14th Annual BeSHG Meeting	Antwerp, Belgium	7/02/ 2014	Making Array CHG technology accessible for Congolese patients using Stripped slides	Poster
BeMGI annual meeting 2014	Liège, Belgium	24th/04/2014	Making array CHG technology accessible for Congolese patients using stripped slides	Poster
25th European Dysmorphology Meeting	Le Bischenberg, Alsace- France	10-12/09/2014	Copy Number Variations in Congolese patients with Intellectual Disability	Oral
15th Annual BeSHG meeting	Palais des Beaux-Arts, Charleroi, Belgium	6/03/2015	Etiological study of intellectual disability in the Democratic Republic of Congo	Poster (Awarded best Poster)
3rd Congress of the CoSHG	Lubumbashi, DR Congo	11-14/05/2015	Etiological study of intellectual disability in the Democratic Republic of Congo	Oral

Courses, Workshops and Seminars

- 08-03-2011: Novel defects in Congenital Disorders of Glycosylation (CDG): looking for the (un)usual aspects! Gert Matthijs, KULeuven
- 17-05-2011: Pacific Biosciences 3rd generation SMRT (tm) single molecule sequencing technology Deepak Singh
- 03-10-2011: Genome evolution, genome instability, Prof Joris Vermeesch, KULeuven
- October 2011 to May 2012: Master English course
- 02-02-2012: Next Generation Sequencing user meeting, Genomics Core, KULeuven
- 29-02-2012: Metastasis signals and pathways (Methusalem Lecture), Prof. Dr. Joan Massagué
- February to May 2012: Master English Course:
- 25-02 to 24-06- 2012: Training Managing my PhD,
- 01-03-2012: Next Generation Sequencing user meeting, Genomics Core, KULeuven
- 06-03-2012: Role of epigenetic enzymes in stem cells and cancer, Prof. Kristian Helin
- 22-03-2012: Single DNA molecule sequencing, Pacific Biotechnology
- 30-03-2012: Why do degenerating neurons die? A look through the fly's eye, Dr. Peter Robin Hiesinger
- 03-04-2012: Candidate gene prioritization by genomic data fusion, Prof. Yves Moreau, KULeuven
- 05-04-2012: Next Generation Sequencing group meeting, Genomics Core
- 08-05-2012: Training Ethics in science,
- April 2012 : Training Health and Safety Environment in Laboratory, Prof Ben Nemery, KULeuven
- 03-05-2012: Next Generation Sequencing group meeting, Genomics Core
- 08-05-2012: Training Ethics in science, Prof. Kris Dierickx, KULeuven
- 07-06-2012: Next Generation Sequencing group meeting, Genomics Core
- 05-06-2012: Molecular mechanisms underlying MR disorders. Prof Ype Elgersma, EMC R'dam
- 28-06-2012: The Ehlers-Danlos syndrome: a disorder with many faces, Fransiska Malfait, UZ Ghent,
- 18-09-2012: Galaxy: open source NGS analyses, Dan Blankenberg from Galaxy
- 28-09-2012: Training in Interpersonal Communications skills, KULeuven
- 09-10-2012: Ethics and Whole Genome Research, by Tim Caulfield'Research
- 09-10-2012: Targeted treatment of Fragile X, Prof Randi Hagerman, MIND institute, University of California, USA Belgium

- 16-10- 2012: Oncogenic pathways and targeted therapies in T-ALL; by Adolfo Terrando: Leuven
- 11-11-2012 to February 2013: Course Statistics for non-statisticians.
- 04-12-2012: Autism and Intellectual Disability: New mutations, Genes and Genetic models Prof Evan Eichler, University of Washington, USA
- 10-12-2012: Towards sequencing everyone's genome, Dr Charles Lee, Harvard Med School Boston
- 10-12-2012: Structural variation and the 1000G project, Dr Charles Lee, Harvard Med School Boston
- 29-01-2013: The genetic basis of major eye malformations, Prof David Fitzpatrick, MRC Edinburgh
- 25-04-2013 Training Note keeping,
- 03-06-2013: Epigenetics and non-coding RNAs in health and disease, Prof Ramin Shiekhattar, The Wistar Institute, USA
- 17 to 20-09-2013: Next Generation Sequencing workshop, Genomics Core, Leuven,
- 08-10-2013: 'Genome-wide mechanisms of BMP/Smad signaling' by Masato Morikawa
- 15-10- 2013: Training qPCR implementation, by Roche's Staff
- 28-10- 2013: 'Dual regulation of axon guidance and cortical spine development by neural
- 07-03-2014: Insights from genetics of neuronal migration disorders and malformations of cortical development (MCD), Prof Dr Jamel Chelly, Institut Cochin, Université Paris Descartes, France
- Training Health and Safety Environment in Laboratory,
- 01 to 03-04 2014: Manchester Dysmorphology Course 2014: Nowgen center, University of Manchester
- 07-04- 2014: Neurofibromatosis Type 1: Manchester story, By Shruti Garg, Manchester, UK
- 09-12-2014: Protein processing in the secretory pathway, Prof John Creemers, KULeuven
- 05-03-2015: Cytoskeletal Mechanisms of Axon growth and regeneration, Frank Bradke, German Center for Neurodegenearative Diseases
- 16-03-2015: Genomic landscapes of histone acetylation in the brain, Prof Angel Barco, Intituto de Neurociencias (UMH-CSIC), Alicante, Spain
- 17-02-2015: Chromosomal mosaicism: from cradle to the grave, Prof Joris Vermeesch, KULeuven
- 12-02-2015: Genetics of Human Brain lateralization, Dr Clyde Franks, Max Planck Institute, Nijmegen, Netherlands
- 12-02-2015: From live imaging to acute protein modification: novel approaches to investigate protein function in living organisms, Markus Affolter, The Center for Molecular Life Sciences, University of Basel, Switzerland

POPULAR SUMMARY

Intellectual Disability (ID) is a frequent medical concern, which affects about 3 % of the world population. In addition, ID has a major social and economic impact on families in developing countries such as DR Congo. Moreover, the attitude of African communities toward ID is influenced by traditional and mystical beliefs. This mostly results in stigmatization and exclusion and increased burden of psychological and social consequences for the individual, parents and families. Therefore, reaching an etiological diagnosis in a person with intellectual disability has an invaluable significance in Africa. In addition, it provides the parents and caregivers with a more accurate prognosis, management plan, allows counseling on recurrence risks.

Both environmental and genetic factors play roles in the etiology of ID, either separately or in combination. Genetic factors causing ID are heterogeneous, and therefore, research into the genetics of ID often associates multiple but complementary strategies. Since some genetic conditions with ID have recognizable clinical manifestations, the first approach is clinically oriented and applies targeted testing based on the clinical hypothesis. The second approach is a screening in patients without clinical clues for a specific diagnosis. With the exception of a few case reports, data on prevalence and types of genetic causes as well as clinical description of central African patients with ID of genetic origin are still missing. In this research, we aimed to contribute to the understanding of the genetic etiology of ID in Congolese patients in Kinshasa, and to investigate the variability of both the clinical presentation and genetic mutations observed.

In the first part of this research, we recruited patients with ID from 6 institutions across Kinshasa and applied a variety of techniques to detect genetic defects that could causal the disability. We were able to identify genetic causes in about one fourth of patients. In many patients, the genetic diagnostic confirmed our clinical suspicion. We did not diagnose Fragile X syndrome in this cohort.

In the second part, we assessed the usefulness of 3 clinical checklists for the selection of patients with a higher risk for Fragile X syndrome. It appeared that the 3 checklists had a high rate of false positive results. This implies that many items included in those checklists, mainly behavioral items, are probably common features among Congolese children with ID, rather than being predictive for Fragile x syndrome as in non-African populations.

An important part of this study focused on the clinical presentation. To date, for the majority of genetic syndromes, the clinical description has been based almost exclusively on studies conducted in Caucasian patients. We wanted to explore whether the presentation of dysmorphic syndromes was

similar between African and Caucasian individuals, and whether clinicians from these 2 settings classify faces of Congolese patients in the same way, meaning either dysmorphic (afwijkend) or not dysmorphic. First, we asked 5 African and 5 European clinicians to score the facial gestalt of Congolese patients. We noticed that, on average, African clinicians considered more patients as dysmorphic (and thus abnormal) compared to European clinicians. It means that the 2 groups of clinicians do not evaluate dysmorphism in the same way. We thought that a more objective phenotyping might clear this difference. Therefore, we tested a recently introduced automated syndrome recognition tool, Face2Gene, in its ability to recognize African versus Caucasian (Flemish) patients with Down syndrome. We found that the application was more efficient in the recognition of Flemish Down syndrome patients than Congolese children with Down syndrome. This means that the variation in clinical presentation may be either a subjective perception by the clinician due to his own ethnic background or a real influence from patients' ethnic background. In addition, reviewing some Congolese cases we diagnosed during our thesis research, we found that further variation in clinical presentation could result from infectious disease, as these are very frequent and may be severely debilitating in a limited resource setting.

Finally, we used the report of a family with Apert syndrome to illustrate how genetic study in Central-African countries may expand our knowledge on disease causing mutations.

This is the first comprehensive study that integrates clinical and molecular genetics on the aetiology of ID in a central African country, the DR Congo. The results of this study show for the first time that genetic factors are a major cause of ID in the DR Congo, as observed in other countries. These results offer the opportunity to openly discuss false believes and feelings of guilt, and establish a firm basis for further research and teaching in the field of genetics of ID.

SAMENVATTING

Verstandelijke beperking (V.B.) is een frequente aandoening die ongeveer 3% van de wereldbevolking treft. De impact van verstandelijke beperking op gezinnen in ontwikkelingslanden zoals de DR Congo is groot, zowel op sociaal als op economisch vlak. Bovendien wordt de houding van de Afrikaanse maatschappij ten opzichte van verstandelijke beperking mee beïnvloed door traditionele en mystieke overtuigingen. Dit resulteert vaak in stigmatisering en uitsluiting, met bijkomende psychologische en sociale gevolgen voor de betrokkene, ouders en families. Daarom heeft het stellen van een etiologische diagnose bij een persoon met een verstandelijke beperking in Afrika een belangrijke betekenis. Bovendien biedt het de ouders en begeleiders een meer nauwkeurige prognose, een betere aanpak en opent het de mogelijkheid van adequate erfelijkheidsadvisering.

Zowel omgevingsfactoren als genetische factoren spelen een rol in de etiologie van verstandelijke beperking, afzonderlijk of in combinatie. De genetische factoren zijn zéér heterogeen en daarom is het genetische etiologisch onderzoek ook gebaseerd op meerdere verschillende strategieën. Gezien sommige genetische aandoeningen klinisch herkenbaar zijn is de eerste benadering steeds klinisch, gevolgd door gerichte tests, gebaseerd op een klinische hypothese. In tweede instantie kan er bij personen zonder klinische aanwijzingen voor een specifieke diagnose genetische screening uitgevoerd worde.

Met uitzondering van enkele case reports, zijn er nagenoeg geen gegevens uit Centraal-Afrika over de genetische oorzaken van verstandelijke beperking en hun prevalentie, maar ook over de klinische manifestaties. Het doel van dit onderzoek was inzicht te verwerven in de genetische oorzaken van verstandelijke beperking bij Congolese patiënten in Kinshasa, en de variabiliteit van zowel de klinische presentatie als genetische mutaties te onderzoeken.

In het eerste deel van dit onderzoek onderzochten we patiënten met een verstandelijke beperking uit 6 voorzieningen in Kinshasa, zowel klinisch als met verschillende genetische tests. Bij ongeveer 1 op 4 van hen kon een oorzaak aangetoond worden, vaak was dit een klinische diagnose bevestigd door genetische testing. Het fragiele X-syndroom werd in deze patiëntengroep niet aangetroffen.

In het tweede deel van ons onderzoek evalueerden we de bruikbaarheid van 3 klinische checklists voor de selectie van patiënten met een hoger risico op het fragiele X-syndroom. Deze 3 checklists hadden een hoog aantal valse positieve resultaten. Dit betekent dat veel kenmerken in deze checklists, en dan vooral items in verband met gedragsproblemen waarschijnlijk frequent voorkomen bij Congolese

kinderen met verstandelijke beperking, en niet zozeer voorspellend te zijn voor het fragiele X-syndroom zoals in niet-Afrikaanse landen.

Een belangrijk deel van ons onderzoek was gericht op de klinische manifestaties. Tot op heden is de klinische beschrijving van de meeste genetische syndromen vrijwel uitsluitend gebaseerd op onderzoek uitgevoerd bij blanke patiënten. Daarom onderzochten we of de presentatie van dysmorfie syndromen vergelijkbaar was tussen de Afrikaanse en Kaukasische individuen, en of Afrikaanse en Europese artsen gezichten van Congolese patiënten op dezelfde manier evalueren aangaande dysmorfie. 5 Afrikaanse en 5 Europese artsen evalueerden onze Congolese patiënten waarbij we merkten dat de Afrikaanse artsen een kind gemiddeld vaker als afwijkend of dysmorf beschouwen in vergelijking met Europese clinici. Dit betekent dat deze 2 groepen artsen dysmorfie niet op eenzelfde manier evalueren. Dit wijst erop dat een meer objectieve fenotypering nodig is. Daarom testten wij een recent geïntroduceerde applicatie voor een geautomatiseerde herkenning van syndromen, Face2Gene. We vonden dat deze applicatie efficiënter was in het correct identificeren van Vlaamse patiënten met het syndroom van Down dan Congolese. Dit betekent dat de variatie in klinische presentatie zowel het gevolg kan zijn van verschillen in subjectieve perceptie door de clinicus als van reële verschillen in presentatie afhankelijk van de etnische achtergrond.

Daarnaast stelden we ook vast dat nadelige omgevingsfactoren zoals infectieziektes een verklaring kunnen zijn van variatie in klinische presentatie, zeker in Centraal Afrika, waar infectieziekten frequent zijn, en gezien de beperkte middelen kunnen leiden tot ernstige verwikkelingen.

Ten slotte illustreren we aan de hand van onze bevindingen in een familie met Apert syndroom, bij wie we een unieke mutatie aantroffen, hoe genetische studies in Centraal-Afrikaanse landen kunnen bijdragen tot onze kennis aangaande genetische mutaties.

Dit is de eerste uitgebreide studie die een klinische evaluatie en (moleculaire) genetische studies combineert om de etiologie van verstandelijke beperking te bestuderen in de DR Congo, een centraal Afrikaanse land. De resultaten tonen voor het eerst dat genetische factoren een belangrijke oorzaak zijn van verstandelijke beperking in de DR Congo, zoals in andere landen. Deze resultaten bieden de mogelijkheid om openlijk verkeerde veronderstellingen en schuldgevoelens te bespreken, en vormen de basis voor toekomstige verder onderzoek en onderwijs in het domein van de verstandelijke beperking.

SCIENTIFIC SUMMARY

Intellectual disability (ID) is a common impairment that affects about 3 % of the world population. Both environmental and genetic causative factors are incriminated. There is an enormous amount of literature on genetics of ID in Western countries. This contrasts with paucity of data from Africa in general and from Central Africa in particular. The prevalence of genetic defects or even the clinical presentation of African patients with a recognized syndromic cause of ID have not been systematically investigated thus far.

This study aimed to contribute to the understanding of the genetic aetiology of ID in Central Africa, to gain insight into the variability in clinical expression of specific genetic disorders in Central Africa, and to gain insight into causes of and variability in genetic mutations observed in Congolese patients with a genetic disorder.

This study was conducted in Kinshasa, the capital of the Democratic Republic of Congo. We collaborated with 6 institutions and recruited 127 patients including 33 (26 %) girls and 94 (74 %) boys with mean age 10.32 ± 4.68 years (range 1.24 - 24.65). After a systematic clinical examination, patients with a suspected diagnosis underwent further specific testing for that disorder. Conversely, patients without a clinical diagnosis were further studied by a genetic screening including microarray-CHG and fragile X syndrome. X-Chromosome Inactivation (XCI) pattern was studied in all available mothers of boys with ID and in all girls, thus selecting patients with a high chance of having an X-linked disorder for Whole Exome Sequencing (WES). WES was also performed in 2 affected sisters, with a suspected autosomal recessive condition.

Overall, we detected a likely genetic cause in 34/127 patients (26.8 %). Down syndrome was the most frequent cause with 19 cases (14.8 %). In 10 out of 86 (11.62 %) patients with ID of clinically unknown aetiology a submicroscopic chromosomal imbalance was detected. We did not identify a single fragile X patient. Of the 41 mothers of boys with ID tested, 3 (7.32 %) (CI: 1.89 % to 18.63%) had a highly skewed X-inactivation (i.e. ≥90/10 %) and among the 27 female index patients with ID, 3 (11.11 %) had also a highly skewed X-inactivation. In one boy, a possible X-linked disorder caused by a variant in the TAF1-gene was identified after exome sequencing. In another child, we identified, by means of exome analysis, a possible autosomal recessive metabolic disorder. The majority of cases (65 %) remain without diagnosis.

Several patients were clinically diagnosed with well-known conditions associated with ID, e.g. Down syndrome, Partington syndrome and Williams syndrome. The features in these conditions were very

similar to those observed in the West. However, we found only a fair agreement between African and European clinicians when evaluating the facial dysmorphism in 127 Congolese patients. Likewise, when we used a recently introduced computed phenotyping program, Face2Gene for the detection of Down syndrome patients, it performed better in Caucasian (80 %) compared to Congolese patients (35 %). Therefore, ethnic background of the patient and of the physician needs to be taken into account during the evaluation of dysmorphism.

The variability in clinical expression may result from environmental factors influencing the course of the disorder. One of the main differences with studies in developed countries is the environment in which Congolese children grow up. Indeed, we also found that in our cohort of patients, infections (such as meningitis) were common. However, in only few of them, an acquired brain insult was convincingly thought to be the cause of their ID. On the other hand, infections may influence the clinical manifestations, therefore one risks to overlook a genetic diagnosis, masked by an intervening environmental insult. For instance, we describe a young boy with the classical facial phenotype of Williams syndrome, caused by the recurrent 1.57 Mb deletion in chromosome 7q11.23. Surprisingly, he had severe ID and absent speech, which is most likely explained by brain damage, secondary to. meningitis and coma at age 2 years. Similarly, we diagnosed a family with X-linked congenital adrenal hypoplasia, where multiple boys died at young age due to infections. The suspicion of a genetic cause was raised when the pedigree revealed an X-linked inheritance pattern.

Currently, very little is known about the causes of mutations. Advanced maternal age is the main risk factor for aneuploidies, and the advanced maternal age observed in the majority of Down syndrome cases in our cohort is in agreement with this. Advanced paternal age is a risk factor for de novo SNV's. However, our study cannot address this question. One of the well-known mechanisms of copy number variations are unequal crossing-overs between low copy repeats (LCR). This results in recurrent, relatively common microdeletions or duplications, which share a same size (flanked by the LCR's) and often a recognisable phenotype. Also in our study, of the 11 submicroscopic imbalances, 4 were recurrent ones. This indicates, not surprisingly, that the same mechanism also operates in the Central-African genome. Also, we report a family with Apert syndrome where a novel mutation was detected, to illustrate that genetic research in Africa can expand our knowledge on the type of mutations in well-characterized genetic disorders.

The results of this study show for the first time that genetic factors are a major cause of ID in the DR Congo. Our study provides local data and insights, which will be useful for teaching and counselling. Obviously, these first results indicate the need for further expansion of similar studies.

WETENSCHAPPELIJKE SAMENVATTING

Verstandelijke beperking is een veel voorkomende aandoening die ongeveer 3% van de wereld bevolking treft. De oorzaak is divers, met zowel omgevingsfactoren als genetische factoren. De grote hoeveelheid literatuur over de genetica van verstandelijke beperking in westerse landen contrasteert sterk met gebrek aan gegevens uit Afrika in het algemeen en vooral van Centraal-Afrika in het bijzonder. De prevalentie van genetische afwijkingen en zelfs de klinische presentatie van Afrikaanse patiënten met klassieke genetische syndromen geassocieerd met verstandelijke beperking is tot nu toe niet systematisch onderzocht.

Deze studie had als doel een bijdrage te leveren aan het begrip van de genetische etiologie van verstandelijke beperking in Centraal-Afrika, om inzicht te krijgen in de variabiliteit in de klinische expressie van specifieke genetische aandoeningen en om inzicht te krijgen in de oorzaken van en de variabiliteit in genetische mutaties waargenomen in Congolese patiënten met een genetische aandoening.

Deze studie werd uitgevoerd in Kinshasa, de hoofdstad van de Democratische Republiek Congo. We werkten samen met 6 instellingen en onderzochten 127 patiënten, waaronder 33 (26%) meisjes en 94 (74%) jongens. De gemiddelde leeftijd was 10,32 ± 4,68 jaar (variërend van 1,24-24,65 jaar). Na een systematisch klinisch onderzoek, werden bij patiënten met een vermoedelijke diagnose verdere gerichte tests voor die aandoening uitgevoerd. Patiënten zonder een klinische diagnose werden verder onderzocht door een genetische screening inclusief microarray-CHG en testing voor het fragiele X syndroom. Het X-chromosoom inactivatie (XCI) patroon werd onderzocht bij alle beschikbare moeders van jongens met verstandelijke beperking en bij alle meisjes, met als doel om patiënten te selecteren met een hoge kans op een X-gebonden aandoening en verdere testing door middel van exoom sequenering (WES). WES werd ook uitgevoerd in 2 zussen met een vermoedelijke autosomaal recessieve aandoening met verstandelijke beperking.

Samengevat werd een waarschijnlijke genetische oorzaak voor de verstandelijke beperking vastgesteld bij 34/127 patiënten (26,8%). Het syndroom van Down is de meest voorkomende oorzaak, vastgesteld bij 19 patiënten (14,8%). Bij 10 van de 86 (11,62%) patiënten met een verstandelijke beperking zonder klinische verklaring werd een submicroscopische chromosomale afwijking vastgesteld. Geen enkele fragiele X patiënt werd geïdentificeerd. Van de 41 moeders van jongens met verstandelijke beperking hadden er 3 (7,32%) een zeer afwijkend X-inactivatie patroon (dwz ≥90 / 10%). Ook bij 3 van de 27 (11.11%) vrouwelijke index patiënten met verstandelijke beperking hadden een zeer afwijkend X-inactivatie patroon. Na exxom sequenering werd bij één jongen, een mogelijke X-gebonden

aandoening geïdentificeerd, veroorzaakt door een variant van de TAF1-gen. Bij een andere patiënt identificeerden we na exoom sequenering een mogelijke autosomaal recessieve stofwisselingsziekte. Bij de meeste patiënten (65%) kon geen oorzaak aangetoond worden.

Bij verscheidene patiënten werden klinisch gediagnosticeerd met een bekende aandoening geassocieerd met verstandelijke beperking, bijvoorbeeld het syndroom van Down, Partington syndroom en het syndroom van Williams. De manifestaties bij deze aandoeningen waren vergelijkbaar met deze waargenomen in het Westen. Nochtans vonden we slechts een matige overeenkomst tussen Afrikaanse en Europese clinici bij het evalueren van faciale dysmorfie bij 127 Congolese patiënten. Ook wanneer we gebruik maakten van Face2Gene, een recent geïntroduceerde applicatie om syndromen te herkennen, bleek dat dit beter geschikt was om Kaukasische syndroom te patiënten correct te herkennen (namelijk 80%) in vergelijking met Congo patiënten (slechts 35%). Deze resultaten tonen aan dat men bij studies aangaande dysmorfie rekening moet houden met de etnische achtergrond van de patiënt maar ook met deze van de arts.

De variatie in klinische expressie kan het gevolg zijn van omgevingsfactoren die het verloop van de aandoening beïnvloeden. Een van de belangrijkste verschillen met de studies in de ontwikkelde landen is de omgeving waarin Congolese kinderen opgroeien. Ook in ons cohort patiënten vonden we dat infecties (bijvoorbeeld meningitis) vaak voorkwamen. Slechts in een paar van hen hadden we voldoende argumenten om een verworven hersenbeschadiging als oorzaak van hun verstandelijke beperking te weerhouden. Anderzijds kunnen infecties ook de klinische manifestaties beïnvloeden, waardoor we het risico lopen om een genetische diagnose te miskennen, omdat deze gemaskeerd wordt door omgevingsfactoren. Zo beschrijven we een jongen met het klassieke lichamelijke fenotype van Williams syndroom, veroorzaakt door de klassieke 1.57 Mb deletie in chromosoom 7q11.23. Verrassend genoeg had hij een ernstige verstandelijke beperking en afwezige spraak, die waarschijnlijk wordt verklaard door een bijkomende hersenbeschadiging, secundair aan meningitis en coma op de leeftijd van 2 jaar. Ook beschrijven we een familie met X-gebonden congenitale bijnier hypoplasie, met meerdere jongens die op jonge leeftijd overleden als gevolg van infecties. Een genetische oorzaak werd gesuggereerd door de stamboom die een X-gebonden overervingspatroon suggereerde.

Momenteel is weinig bekend over de oorzaken van mutaties. Gevorderde leeftijd van de moeder is de belangrijkste risicofactor voor aneuploïdieën, en de gevorderde leeftijd van de moeders in de meerderheid van de Down-syndroom patiënten in onze cohort is hiermee in overeenstemming. Geavanceerde vaderlijke leeftijd is een risicofactor voor de novo SNV's, maar onze studie heeft hier geen gegevens over. Eén van de bekende mechanismes die variaties in het aantal kopijen van bepaalde

chromosoomfragmenten (CNV's) veroorzaken zijn ongelijke crossing-overs tussen low copy herhalingen (LCR's). Dit resulteert in recurrente en dus relatief frequente microdeleties of duplicaties, die meestal eenzelfde grootte hebben en geflankeerd zijn door LCR's, en vaak een herkenbaar fenotype hebben. Ook in onze studie waren 4 van de 11 submicroscopische chromosomale afwijkingen dergelijke recurrente afwijkingen. Niet onverwachte geeft dit aan dat hetzelfde mechanismes ook optreden in het Centraal-Afrikaanse genoom. Ook rapporteren we een familie met Apert syndroom bij wie we een nieuwe mutatie gedetecteerd, hetgeen illustreert dat genetisch onderzoek in Afrika onze kennis kan uitbreiden aangaande type mutaties in goed gekarakteriseerde genetische aandoeningen.

De resultaten van deze studie tonen voor het eerst dat genetische factoren een belangrijke oorzaak van verstandelijke beperking in de DR Congo. Onze studie geeft ons voor het eerst lokale data en inzichten, die nuttig zijn voor het onderwijs en begeleiding. Deze eerste resultaten wijzen op de noodzaak voor verdere, meer uitgebreide en vergelijkbare studies.

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